INSECT VIRUSES AND THEIR USES IN PROTECTING PLANTS

FIELD OF THE INVENTION

The present invention relates to insect viruses useful in control of insect attack on plants. It particularly relates to biological insecticides, especially those comprised of insect viruses. In particular applications, the invention also provides recombinant viruses and transgenic plants.

BACKGROUND OF THE INVENTION

There is increasing awareness of the desirability of insect pest control by biological agents. Considerable effort in recent years has been devoted to the identification and exploitation of DNA viruses with large genomes, especially the baculoviruses. These viruses generally require extensive genetic manipulation to become effective insecticides, and the first such modified viruses are only now being evaluated.

In contrast, very little effort has been devoted to the study and use of small viruses with RNA genomes.

Four main groups of small RNA viruses have been isolated from insects. These include members of the Picornaviridae, the Nodaviridae, the Tetraviridae and the unclassified viruses. Descriptions of these groups can be found in the Atlas of Invertebrate Viruses (eds J.R. Adams and J. R. Bonami) (CRC Press, Boca Raton, 1991) and Viruses of Invertebrates (ed. E. Kurstak) (Marcel Dekker, New York, 1991). These disclosures relating to these viruses concern their pathology and biology, not their use in biological control.

Further information regarding small RNA viruses of insects an be found in P.D. Scotti *et al* (1981) "The biology and ecology of strains of an insect small RNA virus complex" *Advances in Virus Research 26,* 117-143. This review describes the insect picornaviruses cricket paralysis virus and Drosophila C virus (diameters estimated at

27-30 nm with one RNA component of 7.5 - 8.5 kb). N.F. Moore & T.W. Tinsley (1982) The small RNA viruses of insects. Brief review *Archives of Virology 72,* 229-245. This review included viruses of the following families:

Nodaviridae (diameter 29-30 nm, 2 RNA components totalling 4.5 kb)

Picornaviridae (diameter 27-30 nm, one RNA component of 7.5 - 8.5 kb)

Nudaurelia β family (now called Tetraviridae) (diameter around 35 nm, either one RNA of 5.5 kb or two totalling 8 kb)

N.F. Moore, B. Reavy & L.A. King (1985) General characteristics, gene organisation and expression of small RNA viruses of insects. *Journal of general Virology* ***66,*** *647-659.* This reference defines small RNA viruses of insects as being those less than 40 nm in diameter. The review covers Picornaviridae, Nodaviridae and the Nudaurelia β family (now called Tetraviridae).

D. Hendry, V. Hodgson, R Clark and J Newman (1985) Small RNA viruses co-infecting the pine emperor moth (*Nudaurelia cytherea capensis). Journal of general Virology* **66,** 627-632 described viruses with mean diameters of 40nm and 38nm and one or two RNA components up to 5.5 kb in length.

Most recently, the term insect small RNA viruses has been used by one of the present inventors to cover three main recognised toxic groups: the Picornaviridae, the Tetraviridae and the Nodaviridae (P.Scotti & P.Christian (1994) The promises and potential problems of using small RNA insecf viruses for insect control. *Sains Malaysiana 23, 9-18).*

These references illustrate a long standing usage of the term in this field of the term "small RNA virus" for viruses with certain characteristics as listed above. Another important characteristic of these virus groups is that they are not occluded, in contrast to many large viruses like the cytoplasmic polyhydrosis (RNA) viruses or the DNA baculoviruses, granulosis viruses and entomopox viruses. The term would also be applied to viruses not members of the three families listed above, as long as they satisfied the definition of being up to 40nm in size. There are reports of such unclassified viruses (eg in Hendry *et al.* 1985). Moreover, the taxonomic status of some members of the Tetraviridae still requires clarification and it might even be possible for this family to be split, with HaSV and other members with two RNA components in their genome being separated from those with only one component, like the type member *Nudaurelia β* virus, which has not yet been sequenced. The above definition of "small RNA virus" would still cover all members of such virus families.

SUMMARY OF THE INVENTION

In a first aspect of the present invention there is provided an isolated small RNA virus wherein the virus is up to 40nm in size, is not occluded and infects insect species including Heliothis species.

In one particular embodiment, the present invention provides an isolated preparation of *Heliothis armigera* stunt virus referred to as "HaSV" herein.

In a further aspect of the present invention there is provided an isolated nucleic acid molecule comprising a nucleic acid sequence hybridizable with RNA 1 (SEQ ID No: 39) or RNA 2 (SEQ ID No: 47) described herein under low stringency conditions.

In still a further aspect the invention provides a vector comprising a nucleic acid molecule, the sequence of which is hybridizable with RNA 1 (SEQ ID No: 39) or RNA 2 (SEQ ID No: 47) as described herein. These vectors include expression and transfer vectors for use in animals including insect, plant and bacterial cells.

In a further aspect the invention provides an isolated protein or polypeptide preparation of the proteins or polypeptides derivable from the isolated virus of the present invention. The invention also extends to antibodies specific for the protein and polypeptide preparations.

In a yet further aspect the invention provides a recombinant insect virus vector incorporating all or a part of the isolated virus of the present invention.

In a still further aspect of the present invention there is provided a method of controlling insect attack in a plant comprising genetically manipulating said plant so that it is capable of producing HaSV or mutants, derivatives or variants thereof or an insecticidally effective portion of HaSV, mutants, variants or derivatives thereof such that insects feeding on the plants are deleteriously effected. The present invention also provides a transgenic plant so manipulated.

BRIEF DESCRIPTION OF FIGURES

Figure 1a is a restriction map of RNA 1 (SEQ ID No. 39) clones.

Figure 1b is a restriction map of RNA 2 (SEQ ID No. 47) clones,

Figure 2 is the complete sequence of RNA 1 (SEQ ID No. 39) and of major encoded polypeptide.

Figure 3a is the complete sequence of RNA 2 (SEQ ID No. 47) in the authentic version, and its encoded polypeptides.

Figure 3b is the sequence of RNA 2 variant (a 5C version) (SEQ ID No. 51) and its major encoded polypeptide(s).

Figure 4 is bioassay data showing HaSV induced stunting of larvae.

Figure 5 is a map of Vector plasmid pT7T2b and PT7T2c.

Figure 6 is a schematic representation of the proteins encoded by RNA 1 (SEQ ID No. 39) and RNA 2 (SEQ ID No. 47).

Figure 7 is a schematic representation of the proteins expressed by RNA 2 (SEQ ID No. 47) in bacteria DNA

fragments encoding P17 (SEQ ID No. 48), P71 (SEQ ID No. 50), P64, P7 and the fusion protein P70 (SEQ ID No. 52) were synthesized by PCR. The flanking NdeI and BamHI sites used in cloning are indicated. (Note that P17 is followed by BglII site, whose cohesive ends are compatible with those of BamHI).

Figure 8 illustrates the 3'-terminal secondary structure of HaSV RNAs. The tRNA-like structures at the 3' ends of RNAs 1 and 2 (SEQ ID No. 39 & 47) are shown. Residues in bold are common to both sequences.

Figure 9 Expression strategies for HaSV cDNAs in insect cells. The upper part of the figure shows the genome organization of RNAs 1 and 2 (SEQ ID Nos. 39 & 47). The lower part shows insertion of cDNAs corresponding to these RNAs into a plasmid vector, between heat shock protein (HSP70) promoter of Drosophila and a suitable polyadenylation (pA) signal. The HSP promoter was obtained by PCR using suitable primers, with a BamHI site inserted by PCR immediately upstream of the start of the transcription, giving the following sequence: GGATCCACAGnnn (SEQ ID No. 1), where the underlined residue is the transcription start site for either RNA. The cDNAs are terminated by ClaI sites, allowing direct linkage to ribozyme sequences as described in the text.

Figure 10 Ribozymes to yield correct 3' ends. The sequences of ribozymes inserted as short cDNA fragments into HaSV cDNA clones are shown. The ribozyme fragments were assembled and cloned as described in the text. Designed self-cleavage points are indicated by bold arrows.

Figure 11 A-C Immunoblots to map epitopes on HaSV. A. Detected with HaSV antiserum. Lane 1: pTP70delSP; lane 2: pTP70; lane 3: pTP17; lane 4:

control; lane 5: pTP70delN; lane 6: pTP70; lane 7: pTP71; lane 8: HaSV virions; lane 9: molecular weight markers. B. Detected with HaSV antiserum. Lane 1: pTP70delN; lane 2: pTP70 delSPN; lane 3: pTP70. C. Detected with an antiserum to the Bt toxin (CryIA(c)). Lane 1: pTP70; lane 2: HaSV virions; lane 3: control extract.

Figure 12 New field isolates of HaSV. The genomic organization of RNA 2 is shown at the top of the Figure. PCR using appropriate primers with BamHI restriction sites and in some cases altered context sequences of the

AUG initiating translation of the P17 (SEQ ID No. 48) or P71 (SEQ ID No. 50) genes were used to make fragments for cloning into the BamHI sites of the expression vectors. Constructs 17E71 (SEQ ID No. 35) and P71 (SEQ ID No. 50) have altered context sequences of the AUG initiating translation of the P17 (SEQ ID No. 48) and P71 (SEQ ID No. 50) genes respectively; these alterations correspond to the context derived from the JHE gene (see text). All context sequences are given on the right of the Figure. R2 is a clone of the complete RNA sequence as a BamHI fragment in the vector.

Figure 13 Maps of the expression constructs in baculovirus vectors.

Figure 14a to f Various strategies utilizing the present invention.

Figure 15 Expression of RNAs 1 and 2 (SEQ ID Nos. 39 and 47) from baculovirus vectors. The full length cDNA clone of HaSV RNA 1 or 2 (SEQ ID Nos. 39 & 47) was inserted as a BamHI fragment into the baculoexpression vectors. PCR. was used to add BamHI sites immediately adjacent to the 5' and 3' termini of the RNA 1 sequence; sequences of the primers are given in the text. Constructs R1RZ and R2RZ carry cis-acting ribozymes immediately adjacent to the 3' end of the sequence of RNA 1 and 2 (SEQ ID Nos. 39 & 47) respectively.

Figure 16 Expression strategies for HaSV cDNAs in plant cells. The upper part of the Figure shows the

genome organization of RNAs 1 and 2 (SEQ ID Nos. 39 & 47). The lower part shows insertion of cDNAs corresponding to these RNAs into a plasmid vector, between 35S promoter of cauliflower mosaic virus and the polyadenylation (pA) signal on plasmid pDH51 (Pietrzak et al., 1986). The cDNAs were obtained by PCR using suitable primers, with a BaMHI site immediately upstream of the start of each cDNA. The cDNAs are terminated by ClaI sites, allowing direct linkage to ribozyme sequences as described in the text.

DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS

A first aspect of the invention contemplates use of small RNA viruses for biological control of insects. In particular, in accordance with the first aspect of this invention there is provided an isolated small RNA virus, particularly *H. armigera* stunt virus or mutants, variants or derivatives thereof capable of infecting insects, in particular the insect species such as *Helicoverpa armigera*. The small RNA virus isolate of the instant invention is insecticidal and in particular stunts the growth of insect larvae, for example *Helicoverpa armigera* larvae and inhibits or prevents development into the adult stage.

The small RNA viruses of the instant invention have insecticidal, anti-feeding, gut-binding or any synergistic property or other activity useful for insect control.

In particular, *Helicoverpa armigera* stunt virus (HaSV) particles are isometric and approximately 36 nm in diameter with a buoyant density on CsCl gradients of 1.36g/ml. The virus is composed of two major capsid proteins of approximately 64 and 7 KDa in size as determined on SDS-PAGE. The HaSV genome is much later than the largest known nodavirus (another class of RNA viruses) and comprises two ss (+) RNA molecules of approximately 5.3 and 2.4 kb. The genome appears to lack a blockage of unknown structure at the 3' termini that is found in Nodaviridae. The HaSV genome however shares a capped structure and non-polyadenylation with Nodaviridae. HaSV differs significantly from Nodaviridae and Nudaurelia w virus in terms of its immunological properties. In particular the large capsid protein has different antigenic determinants. Other properties of HaSV are described in the Examples.

The host range of HaSV includes Lepidopterans such as from the subfamily Heliothinae. Species known to be hosts are *Helicoverpa (Heliothis) armigera, H. punctigera, H. zea, Heliothis virescens* and other such noctuides as *Spodoptera exigua*. *H. armigera* which is known by the common names corn ear worm, cotton ball worm, tomato grub and tobacco bud worm is a pest of economic significance in most countries. *H.punctigera*, the native bud worm, is a pests of the great economic significance in Australia. Members of the Heliothinae, which include *Helicoverpa* and *Heliothis*, and especially *H.armigera* are among the most important and widespread pests in the world. In the US *Heliothis virescens* and *Helicoverpa zea* are particularly important pests.

The first aspect of the invention provides an isolated small RNA virus capable of infecting insects including *Heliothis* species. In a particularly preferred form the invention relates to mutants, variants and derivatives of HaSV. The terms "mutant", "variant and "derivative" include all naturally occurring and artificially created viruses or viral components which differ from the HaSV isolate as herein described in nucleotide content or sequence, amino acid content or sequence, immunological reactivity, non-glycosylation or glycosylation pattern and/or infectivity but generally retain insecticidal activity. Specifically the terms "mutant", "variant" and "derivative" of HaSV covers small RNA viruses which have one or more functional characteristic of HaSV described herein. Examples of mutants, variants or derivatives of HaSV include small RNA viruses that have different nucleic or amino acid sequences from HaSV but retain one of more functional features of HaSV. These may include strains with genetically silent substitutions, strains carrying replication and encapsidation sequences and signals that are functionally related to HaSV, or strains that carry functionally related protein domains.

In a preferred aspect the invention relates to mutants, variants or derivatives 2of HaSV which encode replication or encapsidation sequences, structures or signals with 60%, preferably 70%, more preferably 80%, still more preferably 90% and even more preferably 95% nucleotide sequence identity to the nucleotide sequences HaSV.

In another preferred aspect the invention relates to mutants, variants or derivatives of HaSV which encode proteins with at least 50%, preferably 60%, preferably 70%, more preferably 80%, still more preferably 90% and even more preferably 95% amino acid sequence identity to proteins or polypeptides of HaSV.

In another preferred aspect the invention relates to mutants, variants or derivatives of HaSV with 50%, more preferably 60%, still more preferably 70%, more preferably 80%, still more preferably 90 or 95% nucleotide sequence identity to the following biologically active domains encoded by the HaSV genome:

RNA 1 (SEQ ID No: 39) - amino acid residues 401 to 600 or the other domains in the replicase

RNA 2 (SEQ ID No: 47) (in the capsid protein)

- amino acid residues 273 to 435
- amino acid residues 50 to 272
- amino acid residues 436 to the COOH terminus

Preferably the viral isolate of the present invention is biologically pure which means a preparation of the virus comprising at least 20% relative to other components as determined by weight, viral activity or any other convenient means. More preferably the isolates are 50% pure, still more preferably it is 60%, even more preferably it is 70% pure, still more preferably it is 80% pure and even more preferably it is 90% or more, pure.

In a second aspect the present invention relates to a nucleotide sequence or sequences hybridizable with those of HaSV. The term nucleotide sequence used herein includes RNA, DNA, cDNA and nucleotide sequences complementary thereto. Such nucleotide sequences also include single or double stranded nucleic acid molecules and linear and covalently closed circular molecules. The nucleic acid sequences may be the same as the HaSV sequences as herein described or may contain single or multiple nucleotide substitutions and/or deletions and/or additions thereto. The term nucleotide sequence also includes sequences with sufficient homology to hybridize with the

nucleotide sequence under low, preferably medium and most preferably high stringency conditions (Sambrook J, Fritsch, E.F. & Maniatis T. (1989) Molecular Cloning: A Laboratory Manual, 2nd Edition, Cold Spring Harbour Laboratories Press) and to nucleotide sequences encoding functionally equivalent sequences. In still a more preferred embodiment the invention comprises the nucleotide sequences of genome components 1 and 2 (SEQ ID Nos: 39 and 47) as represented by Figures 1 and 2 hereinafter or parts thereof, or mutants, variants, or derivatives thereof. The terms "mutants", "variants" or "derivatives" of nucleotide genome components 1 and 2 (SEQ ID Nos: 39 and 47) has the same meaning, when applied to nucleotide sequences as that given above and includes parts of genome components 1 and 2 (SEQ ID Nos: 39 and 47).

The second aspect of the invention also relates to nucleotide signals, sequences or structures which enable the nucleic acid on which they are present to be replicated by HaSV replicase. Furthermore the invention relates to the nucleotide signals, sequences or structures which enable nucleic acids on which they are present to be encapsidated.

In a particularly preferred embodiment of the second aspect, the invention comprises nucleotide sequences which are mutants of the capsid gene having the following sequences:

ATG GGC GAT GCC GGC GTC GCGT TCA CAG (SEQ ID No: 2)
ATG GAG GAT GCT GGA GTG GCG TCA CAG (SEQ ID No: 3)
ATG AGC GAG GCC GGC GTC GCG TCA CAG (SEQ ID No: 4)

In a preferred aspect the invention relates to nucleotide sequences of HaSV encoding insecticidal activity including the capsid protein gene and P17 (SEQ ID No: 48) and mutants, variants and derivatives thereof.

In another preferred aspect the invention comprises nucleotide sequences including the following ribozyme oligonucleotides:

5'CCATCGATGCCGGACTGGTATCCCAGGGGG (called "HVR1Cla" herein) (SEQ ID No: 5)

5' CCATCGATGCCGGACTGGTATCCCGAGGGAC (called "5'HVR2Cla" herein) (SEQ ID No: 6)

5' CCATCGATGATCCAGCCTCCTCGCGGCGCCGGATGGGCA (called "RZHDV1" herein) (SEQ ID No: 7)

5' GCTCTAGATCCATTCGCCATCCGAAGATGCCCATCCGGC (called "RZHDV2" herein) (SEQ ID No: 8)

5' CCATCGATTTATGCCGAGAAGGTAACCAGAGAAACACAC (called "RZHC1" herein) (SEQ ID No: 9)

5' GCTCTAGACCAGGTAATATACCACAACGTGTGTTTCTCT (called "RZHC2" herein) (SEQ ID No: 10)

Ribozyme sequences are useful for obtaining translation, replication and encapsidation of the transcript. It is therefore desirable to cleave the transcript downstream of its t-RNA-like structure or poly A tail prior to translation, replication or encapsidation of the transcript.

The present invention also further extends to oligonucleotide primers for the above sequences, antisense sequences and nucleotide probes for the above sequences and homologues and analogues of said primers, antisense sequences and probes. Such primers and probes are useful in the identification, isolation and/or cloning of genes encoding insecticidally effective proteins or proteins required for viral activity, from HaSV or another virus (whether related or unrelated) carrying a similar gene or similar RNA sequence. They are also useful in screening for HaSV or other viruses in the

field or in identifying HaSV or other viruses in insects, especially in order to identify related viruses capable of causing pathogenecity similar to HaSV.

Any pair of oligonucleotide primers derived from either RNA 1 or RNA 2 (SEQ ID Nos: 39 and 47) and located between ca 300 and 1500 bp apart can be used as primers. The following pairs of primer sequences exemplify particularly preferred embodiments of the present invention: Specifically for RNA 1 (SEQ ID No: 39):

1. HVR1B5' (SEQ ID No: 38) (described below) and the primer complementary to nucleotides 1192-1212 of Figure 1.
2. The primer corresponding to nucleotides 4084 and 4100 of Fig. 13 and the primer HVR13p (SEQ ID No: 12) described below

Specifically for RNA 2 (SEQ ID No: 47):

1. The primer corresponding to nucleotides 459 to 476 of Fig. 2 and the primer complementary to nucleotides 1653 to 1669 of Fig. 2 (this would include the central variable domain)
2. R2cdha5 and the primer complementary to nucleotides 1156 to 1172 of Fig. 2
3. The primer corresponding to nucleotides 1178 to 1194 and the primer complementary to nucleotides 2072 to 2091 (of Fig. 2).

Other combinations giving shorter fragments are also possible.

Further preferred primers include:

5' GGGGGGAATTCATTTAGGTGACACTATAGTTCTGCCTCCCCGGAC (called "HvR1SP5p" herein) (SEQ ID No: 11)

5' GGGGGGATCCTGGTATCCCAGGGGGGC (called "HvR13p"herein) (SEQ ID No: 12)

5' CCGGAAGCTTGTTTTTCTTTCTTTACCA (called "Hr2cdna5" herein) (SEQ ID No: 13)

5' GGGGGATCCGATGGTATCCCGAGGGACGC
TCAGCAGGTGGCATAGG (called "HvR23p" herein) (SEQ ID No: 14)

AAATAATTTTGTTACTTTAGAAGGAGATATACATATGAGCGAGCGAGCACA
C (called "HVPET65N" herein) (SEQ ID No: 15)

AAATAATTTTGTTTAACCTTAAGAAGGAGATCTACATATGCTGGAGTGGCG
TCAC (called "HVPET63N" herein) (SEQ ID No: 16)

GGAGATCTACATATGGGAGATGCTGGAGTG (called "HVPET64N" herein) (SEQ ID No: 17)

GTAGCGAACGTCGAGAA (called "HVRNA2F3" herein) (SEQ ID No: 18)

GGGGGATCCTCAGTTGTCAGTGGCGGGGTAG (called "HVP65C" herein) (SEQ ID No: 19)

GGGGATCCCTAATTGGCACGAGCGGCGC (called "HVP6C2" herein) (SEQ ID No: 20)

AATTACATATGGCGGCCGCCGTTTCTGCC (called "HVP6MA" herein) (SEQ ID No: 21)

AATTACATATGTTCGCGGCCGCCGTTTCT (called "HVP6MF" herein)
(SEQ ID No: 22)

The invention also relates to vectors encoding the nucleotide sequence described above and to host cells including the same. Preferably these vectors are capable of expression in animal, plant or bacterial cell or are capable of transferring the sequences of the present invention to the genome of other organisms such as plants. More preferably they are capable of expression in insect and crop plant cells.

In a preferred aspect the invention relates to the vectors pDHVR1, pDHVR1RZ, pDHVR2, pDHVR2RZ, p17V71, p17E71, pPH, pV71, p17V64, p17E64, pP64, pV64, pBacHVR1, pBacHVR1RZ, pBacHUR2, pBacHVR2RZ, pHSPR1, pHSPR1RZ, pHSPR2, pHSPR2RZ, pSR1(E3)A, pSR1(E3)B, pSR2A, pSR2B, pSX2P70, pSXR2P70, pSRP2B, pBHVR1B, pBHVR2B, pT7T2P64, pSR2P70, pT7T2P65, pT7T2P70, pT7T2-P71, pBSKSE3, pBSR15, pBSR25p, pSR25, phr236P70, phr235P65, pGemP63N, pGemP64N, pGemP65N, pP64N, pP65H, pTP6MA, pTP6MF, pTP17, pTP17delBB, pP656 or p70G as described hereinafter.

In a third aspect the invention relates to polypeptides or proteins encoded by HaSV and to homologues and analogues thereof. This aspect of the invention also relates to derivatives and variants of the polypeptides and proteins of HaSV. Such derivatives and variants include substitutions and/or deletions of one or more amino acids, and amino and carboxy terminal fusions with other polypeptides or proteins. In a preferred aspect the invention relates to the proteins P7, P16, P17 (SEQ ID No: 48), P64, P70 (SEQ ID No: 52), P71 (SEQ ID No: 50), P11a (SEQ ID No: 42), P11b (SEQ ID No: 44), P14 (SEQ ID No: 46) and P187 (SEQ ID No: 40) described herein and to homologues and analogues thereof, including fusion proteins particularly of P71 (SEQ ID No: 50) such as P70 (SEQ ID No: 52) described herein. In a most preferred aspect the invention relates to polypeptides or proteins from HaSV which have insecticidal activity themselves or provide target specificity for insecticidal agents. In particular the invention relates to polypeptides or fragments thereof with insect gut binding

specificity, particularly to the variable domains thereof as herein described. In addition, homologues and analogues with said insecticidal activity of the polypeptides and proteins are also included within the scope of the invention. In addition the invention also relates to antibodies (such as monoclonal or polyclonal antibodies or chimeric antibodies including phage antibodies produced in bacteria) specific for said polypeptide and protein sequences. Such antibodies are useful in detecting HaSV and related viruses or the protein products thereof.

In a fourth aspect the invention provides an infectious, recombinant insect virus including a vector, an expressible nucleic acid sequence comprising all of, or a portion of the HaSV genome, including an insecticidally effective portion of the genome and optionally, material derived from another insect virus species or isolate(s).

Insect virus vectors suitable for the invention according to this aspect, include baculoviruses, entomopoxviruses and cytoplasmic polyhedrosis viruses. Most preferably, the insect virus vector is selected from the group comprising the baculovirus genera of nuclear polyhedrosis viruses (NPV's) and granulosis viruses (GV's). In this aspect of the invention the vector acts as a carrier for the HaSV genes encoding insectidical activity. The recombinant insect virus vector may be grown by either established procedures Shieh, (1989), Vlak (in press) or any other suitable procedure and the virus disseminated as needed. The insect virus vectors may be those described in copending International application No. PCT/AU92/00413.

The nucleic acid sequence or sequences incorporated into the recombinant vector may be a cDNA, DNA or DNA sequence and may comprise the genome or portion thereof of a DNA or RNA of HaSV or another species. The term "material derived from another insect virus species or isolate" includes any nucleic acid sequence, or protein sequence or parts thereof which are useful in exerting an insecticidal effect when incorporated in the recombinant vector of the invention. Suitable nucleic acid sequences for incorporation into the recombinant vector include insecticidally effective agents such as a neurotoxin from the mite *Pyemotes tritici* (Tomalski, M.D.

& Miller, L.K. Nature 352, 82-85 (1991) a toxin component of the venom of the North African scorpion *Androctonus australia* Maeda, S. et al. Virology 184-777-780 (1991) Stewart, L.M.D. et al., Nature 352, 85-88 (1991), Conotoxins from the venom of *Conus spp.* (Olivera B.M. et al., Science 249, 257-263 (1990); Woodward S.R. et al., EMBO J. 9, 1015-1020 (1990); Olivera B.M. et al., Eur. J. Biochem. 202, 589-595 (1991).

The exogenous nucleic acid sequence may be operably placed into the insect virus vector between a viral or cellular promoter and a polyadenylation signal. Upon infection of an insect cell, the vector virus will cause the production of either infectious virus genomic RNA or infectious encapsidated viral particles.

The promoters may be constitutively expressed or inducible. These include tissue specific promoters, temperature sensitive promoters or promoters which are activated when the insect feeds on a metabolite in the plant that it is desired to protect.

Recombinant insect virus vectors according to the present invention may include nucleic acid sequences comprising all or an infectious or insecticidally effective portion of genome the HaSV and optionally another insect virus species or isolate.

In a particularly preferred embodiment of the present invention there is provided assembled capsids comprising one or more of the capsid proteins of the present invention, or derivatives or variants thereof as contemplated or described herein. These assembled virus capsids are useful as vectors for insecticidal agents. As such the assembled viral capsids may be used to administer insecticidal agents such as various nucleotide sequences with insecticidal activity or various toxins to an insect. Nucleotide sequences in the form of RNA or DNA which can be used include those of the HaSV genome or other insect viruses. Toxins which can be used advantageously include those which are active intracellularly and may also include neurotoxins with an appropriate transportation mechanism to reach the insect neurones.

The efficacy or insecticidal activity of infectious genomic RNA or viral particles produced by insect cells infected with insect vectors according to this aspect of the invention, may be enhanced as described below. Moreover the virus vector itself may include within a non- essential region(s), one or more nucleic acid sequences encoding substances that are deleterious to insects such as the insecticidally effective agents described above. Alternatively an extra genome component may be added to the HaSV genome either by insertion into one of the HaSV genes or by adding it to the ends of the genome.

In a particularly preferred embodiment there is provided a recombinant baculovirus vector comprising HaSV or part thereof having insecticidal properties.

Other modifications which may be made to the infectious recombinant insect virus according to the fourth aspect include:

i) splitting the exogenous HaSV nucleic acid molecules comprising the genome and cloning the fragments into the insect vector so that they cannot rejoin. One component, preferably the virus RNA replicase, could be expressed from a separately-transcribed fragment, the transcripts of which would not be replicated by the replicase they encode. The remainder of the genome (having insecticidal activity or encoding the capsid protein or a separate toxin m-RNA) could be encoded by, for example, a second separately-transcribed fragment, the transcripts of which are capable of being amplified by the replicase. Consequently, whilst the transcripts from the second or other fragment would effect their insecticidal activity upon the infected insect cell, they would not be able to infect another insect cell, (even if encapsidated) because the replicase or replicase-encoding transcripts would be absent;

This modification would allow an inherent biological containment to be built into the insecticidal vectors, which, when used in conjunction with the use of non-persistent DNA virus vectors such as those described in the above

mentioned copending application, would allow a new level of environmental safety greatly extending earlier approaches based on baculovirus vectors.

ii) Manipulation of encapsidation signals or sequences essential for replicase binding or production of sub-genomic mRNA's including expression of exogeneous insect control factors as RNAs dependent on the virus for replication. This involves determination of RNA sequences and signals important for replication and encapsidation of virus RNAs, such as by analysis of replication of deletion mutants carrying reporter genes in appropriate cells, followed by studies on the transmission of the reporter gene to larvae by feeding of virus. These deletion mutants can be used to carry genes for insect control factors/toxins to larvae after replacing the reporter gene by a suitable toxin gene such as shown in Fig. 12;

iii) using an insect promoter responsive to virus infection and, for example, placing copies of the viral replicase gene under the control of two promoters, one which is constitutive or expressed at early stages of vector infection, and the other being a cellular promoter turned on by the ensuing RNA viral infection. This system would then make more copies of the replicase mRNA available as the amount of its template increased. Such a promoter may be isolated using techniques analogous to enhancer trapping, that is, transforming insect cells with a suitable reporter gene and looking for induction of the reporter upon virus infection of a population of transformed cells.

In a fifth aspect the invention relates to a method of controlling insect attack in plants by genetically manipulating plants to express HaSV or parts thereof which can confer insecticidal activity optionally in combination with other insecticidally effective agents. Such plants are referred to as transgenic plants.

The term "express" should be understood as referring to the process of transcribing the genome or portion thereof into RNA or, alternatively, the process of transcribing the

genome or portion thereof into RNA and then, in turn, translating the RNA into a protein or peptide.

In a sixth aspect the invention relates to the transgenic plants per se as described above. Transgenic plants according to the invention may be prepared for example by introducing a DNA construct including a cDNA or DNA fragment encoding all or a desired infectious portion of HaSV, into the genome of a plant. The cDNA or DNA fragment may, preferably, be operably placed between a plant promoter and a polyadenylation signal. Promoters may cause constitutive or inducible expression of the sequences under their control. Furthermore they may be specific to certain tissues, such as the leaves of a plant where insect attack occurs but not to other parts of the plant such as that used for food. The inducible promoters may be induced by stimuli such as disturbance of wind or insect movement on the plant's tissues, or may be specifically turned on by insect damage to plant tissues. Heat may also be a stimulus for promoter induction such as in spring where temperatures increase and likelihood of insect attack also increases. Other stimuli such as spraying by a chemical (for instances a harmless chemical) may induce the promoter.

The cDNA or DNA fragment may encode all or a desired infectious portion of the wild-type, recombinant or otherwise mutated HaSV. For example, deletion mutants could be used which lack segments of the viral genome which are non-essential for replication or perhaps pathogenicity.

The nucleotide sequences of the invention can be inserted into a plant genome by already established techniques, for example by an Agrobacterium transfer system or by electroporation.

Plants which may be used in this aspect of the invention include plants of both economic and scientific interest. Such plants may be those in general which need protection against the insect pests discussed herein and in particular include tomato, potato, corn, cotton, field pea and tobacco.

To enhance the efficacy of infectious genomic RNA or viral particles expressed by transgenic plants according to the invention, the DNA construct introduced into the plants' genome may be engineered to include one or more exogenous nucleic acid sequences encoding substances that are deleterious to insects. Such substances include, for example, *Bacillus thuringiensis* d-toxin, insect neurohormones, insecticidal compounds form wasp or scorpion venom or of heterologous origin, or factors designed to attack and kill infected cells in such a way so as to cause pathogenesis in the infected tissue (for example, a ribozyme targeted against an essential cellular function).

DNA constructs may also be provided which include:

i) mechanisms for regulating pathogen expression (for example, mechanisms which restrict the expression of ribozymes to the insect cells) by tying for example, expression to abundant virus replication, production of minus-strand RNA or sub-genomic mRNA's; and/or

ii) mechanisms similar to, or analogous to, those described in copending International patent application number PCT/AU92/00413 so as to achieve a limited-spread system (such as control of replication).

Transgenic plants according to the present invention may also be capable of expressing all or an infectious or insecticidal portion of genomes from HaSV and one or more species or isolates of insect viruses.

In a seventh aspect of the invention HaSV, or insecticidally effective parts thereof, or the infectious recombinant virus vectors of the fourth aspect of the present invention may be applied directly to the plant to control insect attack. HaSV or the recombinant virus vectors may be produced either in whole or in part in either whole insects or in culture cells of insects or in bacteria or in yeast or in some other expression system. HaSV or the recombinant virus forms may be applied in a crude form, semi purified or

purified form optionally in admixture with agriculturally acceptable carrier to the crop in need of protection. HaSV may also be applied as a facilitator of infection where existing insect populations already infected with another agent, such as one or more other viruses whereby HaSV is able to act synergistically to bring about an insecticidal effect. Alternatively HaSV and another agent such as one or more viruses may be applied together to plants to control insects feeding thereon.

A deposit of HaSV No. 18.4 was made on August 5th 1992 at the Australian Government Analytical Laboratories. The deposit was given accession No. N92/35575.

EXAMPLE 1

TAXANOMIC, PHYSIOCHEMICAL AND BIOCHEMICAL CHARACTERISATION OF AN INSECT VIRUS: HaSV

Materials and Methods

A **Animals and virus production.** H. Armigara larvae were raised as described in Teakle R.E. and Jensen J.M. (1985) *Heliothis punctiger* in Singh P and Moore R.F. (eds) Handbook of Insect Rearing Vol 2., Elsevier, Amsterdam pp 313-322. Larvae were infected for virus production by feeding five day old larvae on 10mg pieces of diet to which 0.064 OD_{260} units of HaSV had been applied. After 24 hours the larvae were then transferred to covered 12-well plates (BioScientific, Sydney, Australia) that contained sufficient diet and grown for eight days after which they were collected and frozen at -80°C until further processed. Frozen larvae were weighed to 100g, placed into 200ml of 50mM Tris buffer (pH 7.4), homogenized, and filtered through four layers of muslin. This homogenate was centrifuged in a Sorvall SS-34 rotor at 10,000 x g for 30 minutes whereupon the supernatant was transferred to fresh tubes and recentrifuged in Beckman SW-28 rotor at 100K xg for 3 hours. The resultant band was collected and repelleted in 50 mM pH 7.2 Tris buffer in a Beckman SW-28 tube by centrifugation at 100K xg for 3 hours. The pelleted virus was resuspended overnight in 1ml of buffer at 4°C then layered onto a

discontinuous CsCl gradient containing equal volumes of 60% and 30% CsCl (w/v) in a Beckman SW-41 tube and centrifuged at 12 h at 200 xg. The resultant pellet was suspended in 100ml of buffer and frozen for further use.

B **Particle characterization.** Staining with acridine orange was as described in Mayor H.D. and Hill N.O. (1961) Virology 14: p264. Buoyant density was estimated in CsCl gradients according to Scotti P.D., Longworth J.F., Plus N, Crozier G. and Reignanum C. (1981) Advances in Virus Research 26: 117-143.

C **Immunological procedure.** Rabbit anti-sera to HaSV was produced by standard immunological procedures. Rabbit antisera to the Nudaurelia o virus in addition to the virus itself was provided by Don Hendry (Rhodes University, Grahamstown, South Africa). Rabbit antisera to the Nudaurelia b virus was supplied by the late Carl Reinganum (Plant Research Institute, Burnley, Vic, Australia). The immunological relationship to the Nudaurelia w virus was determined by the standard reciprocal double diffusion technique. Immunoblotting was performed according to Towbin H., Staeheln T. and Gordon J. (1979) PNAS. Antibodies monospecific for the major 65 kDa capsid protein were prepared by incubating polyclonal antisera with sections of nitrocellulose blotted with the 65 kDa protein. After extensive washing in Tris buffered saline, the bound antibodies were eluted in 50mM citric buffer, pH 8.0 after a 5 minute incubation.

D **Protein characterization.** Polyacrylamide gel electrophoresis in the presence of SDS followed the procedure of Laemmli UK 1970 Nature 227: 680-685 and was done with 12.5% gels unless otherwise noted with low and high molecular weight standards from BioRad. Staining was done with a colloidal preparation of Coomassie Blue G-250 (Gradipore Ltd, Pyrmont, New South Wales, Australia). Determination of the M_r of the smallest protein was done with a 16% gel and standards of 3.4 kDa, 12.5 kDa and 21.5 kDa (Boehringer Mannheim). Glycosylation of the viral proteins was determined by a general

glycan staining procedure with reagents supplied by Boehringer Mannheim; the positive control was fetuin. N-termini of proteins were sequenced using procedures described by Matsudairia (1989) Purification of Proteins and Peptides by SDS-PAGE in A Practical Guide to Protein and Peptide Purification for Microsequencing ed Matsudaira P.T. Academic Press, San Diego pp 52-72 on an Applied Biosystems 477A gas phase sequencer.

Nucleic acid characterization. RNA was removed from capsids by twice vortexing a virus suspension with equal volumes of neutralized phenol then with phenol/chloroform (50:50). RNA was then precipitated from the aqueous phase in the presence of 300 mM sodium acetate and 2.5 volumes of ethanol. Digestions of the HaSV nucleic acid with RNAse A and DNAse I (Boehringer Mannheim) were done with pBSSK(-) phagemid ssDNA and dsDNA (Stratagene) and RNA controls (BRL). Denaturing agarose gel electrophoresis in the presence of formaldehyde was performed according to Sambrook et al (1989). The state of polyandenylation of the viral RNA was determined by two methods. The first method was to compare the binding of identical amounts (20 mg) of viral RNA and poly(A)-selected RNA from *Helicoverpa virescens* to a 1ml slurry of 5mg of oligo-d(T) cellulose (Pharmacia) in a binding buffer consisting of 20 mM Tris pH 7.8, 500 mM NaCl, 1 mM EDTA and 0.04% SDS. The second method was to observe specific priming of viral RNA and viral RNA polyadenylated with poly(A) polymerase (Pharmacia) with $d(T)_{16}A/C/G$ primers in RNA sequencing reactions using reverse transcriptase (US Biochemical) and a protocol provided by the supplier. The 5' cap structure of the genomic RNA and HaSV was determined by observing the ability of polynucleotide kinase to phosphorylate viral RNA with and without preincubation with tobacco acid pyrophosphatase and alkaline phosphatase (Promega) under conditions described by the supplier.

F ***In vitro* translation of HaSV RNA.** *In vitro* translation of HaSV RNA was performed with lysates of both rabbit reticulocytes and wheat germ (Promega) as directed by the supplier. Reactions were conducted in 10 ml volumes with 1.0 mg of RNA in the presence of five u Ci ^{35}S-methionine. The labelled proteins were resolved on 10% and 14% SDS-PAGE gels as described above then visualised by autoradiography of the dried gels. The two viral RNAs were separated by a "freeze and squeeze" method after resolution on nondenaturing low-melting-point agarose gels in TAE (Sambrook, et al. 1989). Briefly, agarose slices containing the RNA were melted at 65° C in a volume of TAE buffer equal to six times the agarose volume. The solution was allowed to gel on ice before freezing at -80° C for 30 minutes. The frozen solution was thawed on ice then centrifuged at 14,500xg for 10 minutes after which the supernatant was withdrawn and precipitated by the addition of ethanol.

G **Bioassay of virus-induced pathogensis**

Known amounts of virus isolate, as shown in Figure 3, were fed to larvae at the growth stages indicated by admixture to stadnard diet. At the time points shown, the larvae were weighed and the mean and SD calculated. Growth of infected larvae was compared to those of uninfected control populations from the same hatching batch in every experiment.

Results

i) Characteristics and taxonomy of HaSV

The virus particles are isometric and are approximately 36 - 38 nm in diameter. They are composed of two major capsid proteins, of 65 kDa and 6kD is size. The virions contain two single-stranded (+) RNA species of 5.3 kb and 2.4 kb length. The virus bears a similarity in these respects to the Nudaurelia w virus, which has been tentatively regarded as a member of the Tetraviridae; these two viruses differ however,

in the above respects from other viruses in this group and are likely to form a new virus family, sharing chiefly their capsid structure (T=4) with the Tetraviridae.

ii) Particle characterization and serology.

The buoyant density of HaSV was calculated to be 1.296g/ml in CsCl at pH 7.2. The A_{260}/A_{280} ratio of HaSV viral particles was 1.22 indicating a nucleic acid content of approximately 7% (Gibbs and Harrison, (1976) Plant Virology: The Principles London: Edward Arnold. Reciprocal immuno-double diffusion comparisons between HaSV and the Nudaurelia w virus showed no serological relationship. The more sensitive technique of immunoblotting also showed a complete lack of any antigenic relationship. In addition, HaSV did not react with antisera to the Nudaurelia b virus in a immuno-diffusion test or when immunoblotted. However, no Nudaurelia b virus was available as a positive control in these latter two immunological experiments. When HaSV was stained with acridine orange then irradiated with 310nm UV light, the particles fluoresced red which indicated a single stranded genome.

iii) Protein characterization.

Examination of the capsid proteins of HaSV with polyacrylamide gel electrophoresis in the presence of SDS showed variable results depending on the quantity of protein present. At low protein loadings, two proteins in major abundance were evident that had M_r's of 65,000 and 6,000 along with a protein in minor abundance with M_r of 72,000 (data not shown). When more protein was present on the gels, however, at least 12 more distinct bands with M_r's ranging between 15,000 and 62,000 became evident. Probing the resolved and blotted proteins with antibodies monospecific for the major 65 kDa capsid protein showed all but two of the proteins shared common antigens with the major 65 kDa protein. The major 6 kDa capsid protein and a minor band migrating at M_r=16,000 failed to react with both the monospecific antibodies and untreated antisera.

The capsid proteins were shown to be non-glycosylated as they failed to react with a hydrazine analog after oxidation with periodic acid. The N-terminus of the 65 kDa protein appeared to be blocked in some manner as two efforts to conduct an Edman degradation failed. After the second attempt, the sample was treated with n-chlorosuccinimide and shown to be in a quantity normally adequate for sequencing. The N-terminus of the 6 kDa protein, however, was not blocked as an unambiguous 16-residue sequence was readily obtained. The sequence of the N-terminus of the 6 kDa capsid protein and those of a cyanogen bromide cleaved fragment of the 65 kDa protein are as follows:

6 kDa protein:
PheAlaAlaAlaValSerAlaPheAlaAlaAsnMetLeuSerSerValLeuLysSer
(SEQ ID No: 23)

65 kDa protein:
ProThrLeuValAspGlnGlyPheTrpIleGlyGlyGlnTyrAlaLeuThrProThrSer
(SEQ ID No: 24)

Detailed sequence analysis of the RNA genome carried out in Example 3 showed that RNA 1 (SEQ ID No: 39) encodes a protein of molecular weight 186,980 hereinafter

referred to as P187 (SEQ ID No: 40) and RNA 2 (SEQ ID No: 47) encodes proteins with molecular weight 16, 522 (called P17 (SEQ ID No: 48)) and 70,670 (called P71 (SEQ ID No: 50)). P71 (SEQ ID No: 50) is processed into two proteins of molecular weight 63,378 (called P64) and 7,309 (called P7).

iv) Nucleic acid characterization

The extracted nucleic acid from HaSV was readily hydrolysed by RNAse A but not by DNAse I. Denaturing agarose gel electrophoresis of the extracted RNA genome of HaSV indicated two strands that migrated at 5.5 kb and 2.4 kb. The RNA strands were shown not to have extensive regions of polyadenylation as only 24% of the viral RNA bound to the oligo-d(T) cellulose matrix as opposed to 82% of poly(A)-selected RNA. Further evidence for the non-polyadenylation of the viral genome was provided by the observation that the oligo primer, $d(T)_{16}G$, gave a clear sequencing ladder using reverse transcriptase only after *in vitro* polyadenylation of the viral strands with poly(A)-polymerase.

The demonstration that the strands could be modified with poly(A)-polymerase also showed the lack of any 3' modification. The 5' termini of the viral strands were shown to be capped, most likely with $m^7G(5')ppp(5')G$, as they could not be labelled with polynucleotide kinase unless pretreated with tobacco acid pyrophosphatase and alkaline phosphatase.

v) *In vitro* translation.

In vitro translation of the viral RNA yielded different results in the two translation systems used (data not shown). The 5.5 kb RNA translated very poorly in the reticulocyte system whereas it produced in the wheatgerm system more than 20 proteins ranging in size from M_r=195,000 to M_r=12,000. The 2.4 kb viral RNA strand yielded a major protein with an M_r=24,000 in both systems in addition to a minor protein at M_r=70 kDa. A time course of the translation reaction with the 5.5 kb RNA strand showed all labelled proteins were produced at similar rates indicating that the

smaller products did not arise through processing of the larger ones. However when a time course experiment was done with translation of the smaller 2.4 kb RNA strand, the 24 kDa protein appeared before the 70 kDa protein.

vi) Presence of another form of HaSV

Frequently, during purification of HaSV virions, a minor band appeared in varying amounts on the CsCl gradient that had a buoyant density of 1.3 g/ml. On four occasions, when particles from this minor band were used to infect *H. armigera* larvae that were then processed as before for purification of HaSV virions, the HaSV band with a density of 1.296g/ml was again recovered in vast excess to a varying minor amount of the more dense band. No virions of either type were recovered from uninfected control larvae. Proteins extracted from the more dense particles appeared identical to those from the less dense particles when examined by SDS-PAGE and immunoblotting with antibodies specific for the 65 kDa capsid protein of HaSV. Extraction and examination of the RNA genome with denaturing agarose gel electrophoresis also showed the same 5.5 and 2.4 kb bands. When particles from the more dense band were examined by electron microscopy as before, they appeared to have a larger diameter 45nm but otherwise highly similar to the 38nm particles.

The molar ratio of the two RNA strands was determined by quantitative densitometry of fluorograms of the resolved strands. The ratio derived from an average of four measurements of various loadings on denaturing gels proved to be 1.7:1 (5.5 kb strand: 2.4 kb strand) which is somewhat lower than the expected ratio of 2.3:1 for equimolar amounts of each strand.

The genome of HaSV has major differences that make it distinct from those of the nodaviruses, the only other group of bipartite small RNA viruses pathogenic to animals. Although HaSV shares the characteristic of a bipartite genome with the only animal viruses having such a divided genome, the nodaviridae, it differs in virtually every other aspect from this group. Both segments of its genome are considerably larger than the

corresponding nodaviral RNAs (Hendry D.A., (1991) Nodaviridae of Invertebrates. in (ed. E. Kurstak) Viruses of Invertebrates. Marcel Dekker, New York, pp. 227-276). However, the division of genetic labour is similar with the larger component carrying the replicase gene and the smaller one encoding the capsid proteins. Direct comparison of the sequences shows little homology between these viruses, at either RNA or protein level. The Nodaviruses, have the already mentioned unusual 3'blockage (probably a protein), whereas the HaSV RNAs terminate in a distinctive secondary structure resembling a tRNA.

vii) Bioassays of virus isolates on larvae

The original constructs made to express the capsid proteins (precursor and processed forms) in *E. coli* for bioassay started at the first AUG (nts 284 to 286). Production of full-length, immuno-reactive protein from these was due to these clones being the 5C sequence version with the extra C residue. Bioassays of these proteins have been difficult due to problems with obtaining suitable *Heliothis* larvae for the tests.

Purified native HaSV was used to conduct bioassays in non-noctuid insect species. The native HaSV was orally administered, the larvae scored for symptoms of infection and growth was measured. Dot blotting for HaSV RNA was also conducted. Based on these experiments native HaSV does not appear to infect the following larvae.

Species	**Order**	**Family**
Galleria mellonella	Lepidoptera	Pyradidae
Tineola bissellia	Lepidoptera	Tineidae
Epiphyas postvittana	Lepidoptera	Tortricidae
Lucilia cuprina	Diptera	Calliphoridae
Dacus tyronii	Diptera	Tephritidae
Antitrogus parvulus	Coleoptera	Scarabaedae
Lepidiota picticollis	Coleoptera	Scarabaedae
Sericesthis germinata	Coleoptera	Scarabaedae

The above experiment conducted with the larvae of *Spodoptera exigua* and *S. litura* showed that native HaSV infects these species but not to the same degree as seen in *Heliothis armigera*.

EXAMPLE 2

OTHER VIRUS ISOLATES

Materials and Methods

A Virus isolation

Apparently infected (*viz* diseased) larvae of *Helicoverpa sp* were collected in February 1993 at Mullaley (NSW), Narrabri (NSW) and Toowoomba (QLD) (Australia). Referring to Fig. 10 the samples in wells 2A-2D were from parasitised *H. armigera* larvae collected from sorghum at Mullaley; the sample in 6C was collected from sunflower at Toowoomba; the sample in 7D was collected from cotton at the Narrabri Research Station. The latter two larvae may have been either *H. armigera* or *H. punctigera*, which are both easily infected with HaSV.

B Virus RNA Extraction

Larvae collected were ground up and RNA extracted. RNA extraction and purification were as per Example 1.

C Dot-Blot Northern Hybridization

Extracts of viral RNA was analysed by Northern dot-blot hybridisation using a probe made from cloned HaSV sequences derived from 3'-terminal 1000 units of RNA 1 and RNA 2 by random priming in a Boehringer Mannheim kit according to the supplier's instructions were employed. RNA extracts were transferred to Zeta-Probe (BioRad) for probing. Hybridization under high stringency washing conditions were as specified by BioRad. Hybridizations were carried out in the following solution:

1 mM EDTA, 500 mM HaH_2PO_4, pH 7.2, 7% SDS, at 65°C in a rotating Hybaid hybridization chamber. After completion of hybridization and removal of the solution containing the probe, the

filters were washed twice in l mM EDTA, 40 mM HaH_2PO_4 pH 7.2, 5% SDS, at 65°C (l h each), followed by 2 washes in l mM EDTA, 40 mM HaH_2PO_4, pH 7.2 l% SDS, at 65°C (1 h each), before autoradiography.

RESULTS

Referring to Fig. 10, samples 9A, 9B, 10A, 10B and 10C contain HaSV infected positive control lab-raised larvae; 9C-H contain healthy (HaSV-free) negative control lab-raised larvae; All other wells (beginning 1-8) contain extract from field-collected larvae. Numbers 2A-D, 6C and 7D gave positive signals indicating that these isolates are either the same as HaSV or derivatives or variants thereof. Election microscopy employing (-) staining confirmed that the samples which gave positive signals contained abundant icosohedral virus particles of approximately 36mm in size.

The presence of HaSV in larvae which had tested positive in the Northern hybridization dot-blot was confirmed by Western blotting of crude extracts from such infected larvae, using the polyclonal antibody to the HaSV capsid protein. For routine screening of such extracts in order to identify further isolates of HaSV or to confirm the presence of the virus, use of a monoclonal antibody or its equivalent is preferable, in order to achieve (i) higher sensitivity of detection and (ii) greater specificity of detection.

EXAMPLE 3

IDENTIFICATION, ISOLATION AND CHARACTERISATION OF INSECT VIRUS GENES

Materials and Methods

A Animals and virus production.

H. armigera larvae were raised as described in Example 1.

B Protein characterization

Was conducted as described in Example 1.

C Nucleic acid characterization

Was conducted as in Example 1.

D Fractionation of virus RNA

The two viral RNAs were separated by a "freeze and squeeze" method after resolution on nondenaturing low melting point agarose gels in TAE (Sambrook, et al, 1989). Briefly, agarose slices containing the RNA were melted at 65° C in a volume of TAE buffer equal to six times the agarose volume. The solution was allowed to gel on ice before freezing it at -80° C for 30 minutes. The frozen solution was thawed on ice then centrifuged at 14,500g for 10 minutes after which the supernatent was withdrawn and precipitated by the addition of ethanol.

E *In vitro* translation of HaSV RNA

Was as in Example 1.

F cDNA synthesis and cloning of virus genome

The virus RNAs were reverse transcribed into cDNA using the Superscript RTase (a modified form of the Moloney murine leukaemia virus (MMLV) RTase, produced by Life Technologies Inc). Oligo(dT) was used as a primer on RNA which had been polyadenylated *in vitro*. After size selection of DNA fragments over 1 kbp in length, the cDNA was then blunt-end ligated using T4 DNA ligase (Boehringer Mannheim or Promega, under conditions described by the suppliers) into vector pBSSK(-) (Stratagene) which had been cut with EcoRV and dephosphorylated with calf intestinal alkaline phosphatase (Boehringer Mannheim). E.coli strain JM109 or JPA101 were electroporated with the ligation mixture and white colonies selected on colour-indicator plates Sambrook et al, 1989.

For some clones of RNA2 (SEQ ID No: 47), cDNA was synthesised using the RTase of AMV (Promega) and a specific primer complementary to nucleotide sequence 2285

- 2301 of RNA 2 (SEQ ID No: 47). The same buffer and conditions were used for the Superscript RTase (above). The AMV RTase was found not to make cDNA form a primer annealing to the terminal 18 nucleotide sequence (see below), nor to be able to reach the 5'-end of the RNA with the primer here described.

G Sequencing of DNA and RNA

The cDNA clones were separated as single-stranded or double-stranded DNA, using the deaza-dGTP and deaza-dITP nucleotide analogues (Pharmacia) in the deaza T7 sequencing kit as recommended by this supplier. Synthetic oligonucleotides were used as primers. The 5' terminal sequences of the two RNAs were determined using reverse transcriptase to sequence the RNA template directly, from specific oligonucleotide primers located about 200 nucleotides downstream from the termini. Such RNA sequencing was performed using the reverse transcriptase sequencing kit from Promega, under the conditions described by the manufacturer.

The sequence of the 20 or so nucleotides at the 5' terminus of each RNA was checked using direct RNase digestion of 5'-labelled RNA under conditions designed to confer sequence-specificity. Direct RNA sequence using RNases was performed with the RNase sequencing kit from US Biochemicals, following the protocols provided by the manufacturer. This also confirmed that the sequence of the most abundant RNA is consistent with that of the RNA analysed using the specific primer and RTase.

All transcription of plasmids linearized as described were performed as recommended by the suppliers of SP6 RNA polymerase, in the presence of 1mM cap analogue, 0.2mM GTP, and 0.5mM of the other NTPs.

H Subcloning and expression

PCR *amplification*

The polymerase chain reaction (PCR) was used to obtain sequences covering virus genes in a form suitable for cloning into expression vectors. The reaction was performed with Taq DNA polymerase (Promega) as described by the supplier, in a rapid cycling thermal sequencer manufactured by Corbett Research (Sydney, Australia). A typical reaction involved 1 cycle of 1 min at 90°C, 25 cycles of 95°C (10 sec), 50°C (20 sec), 72°C (1.5 min), followed by one cycle of 72°C for 5 min. Templates were generally cDNA or cDNA clones derived from HaSV RNAs, made as described below. Primers were as described below for the relevant constructs.

Upon termination of the PCR reaction, the product's ends were made blunt by treatment with E.coli DNA polymerase I (Klenow fragment) at ambient temperature for 15 minutes. After heating at 65° C for 10 minutes, the reaction was cooled on ice and the reaction mix made 1mM in ATP. The product then 5'-phosphorylated using 5 units of T4 polynucleotide kinase at 37° C for 30 minutes. After heating at 65° C for 10 minutes, the product was run on a 1% low-melting agarose gel and purified as described for RNA in section E above.

ligations: Vectors and restriction fragments cut with the enzymes described were run on 1% low-melting-point agarose gels and excised as slices. These slices were then melted at 65° C for 5 minutes, before cooling to 37° C. Fragment and vectors were then ligated in 10ul total volume at 14° C overnight using T4DNA ligase (BRL, Boehringer Mannheim or Promega), in the buffers supplied by the manufacturers.

expression: Expression plasmids containing viral genes (e.g. for the capsid protein) were transformed into *E. coli* strain BL21 (DE3) or HMS174 (DE3) (supplied by Novagen). After growth as specified by the supplier, protein expression was induced by the addition of isopropyl b-D-thiogalactopyranoside (IPTG), at 0.4 nM to the growing culture for a period of 3h. Expressed proteins were analysed by SDS-polyacrylamide gel electrophoresis of bacterial extracts (Laemmli, 1970).

Results

i) Mapping cDNA clones of HaSV

The template for cDNA synthesis was virus RNA which had been polyadenylated in vitro. Oligo(dT) was used as a primer for the Superscript reverse transcriptase (RTase; a modified form of the Moloney murine leukaemia virus (MMLV) RTase, produced by Life Technologies Inc). The cDNA was cloned into vector pBSSK(-) as described earlier. The larger clones were selected for further analysis by restriction mapping and Northern hybridization. All the probes tested hybridized either to RNA 1 or to RNA 2, suggesting that there are no regions of extensive sequence homology between the two RNA's. Furthermore, screening of a number of other clones excluded the theoretical possibility that either RNA band may actually contain more than one species.

ii) RNA 1 (SEQ ID No: 39) clones

Three large RNA 1 (SEQ ID No: 39) clones (B11U, B11O and B35) obtained for the first round of clones were further analysed by restriction mapping and shown to form an overlap spanning over 3 kbp (this was later confirmed by sequencing). The second round of cloning then yielded E3 of 5.3 kbp, representing 99.7% of RNA 1 (SEQ ID No: 39). A complete restriction map of clone E3 showed it to align with that previously determined for three overlapping clones. On the basis of this alignment, the 5' end of the insert in B11U was placed about 300 nucleotides downstream from the 5' end of the RNA.

Once clones covering a contiguous block had been identified, the orientation 3relative to the RNA was determined.

iii) RNA 2 (SEQ ID No: 47) clones

Three significant cDNA clones were isolated for RNA 2 (SEQ ID No: 47) (Fig. 2). One, hr236, contains about 88% of RNA 2 (SEQ ID No: 47) (2470 bp total length), and runs from the 3' end to 240 bp from the 5' end. The other clones, hr247 and hr 249

position 34 and terminates at nucleotide 5290 and is thought to encode the RNA-dependent RNA polymerase (replicase)(referred to as P187 (SEQ ID No: 40) in Fig. 1) required for virus replication, since it contains the Gly-Asp-Asp conserved triplet and surrounding sequences identified in these enzymes, which are usually large (over 100 kDa), in addition to further homology with the polymerase encoded by tobacco mosaic virus and other plus-stranded RNA viruses.

Referring to Fig. 1 the sequence is presented as the upper strand of the cDNA sequence. This strand is therefore in the same sense as the viral (positive-sense) RNA. The sequence of the protein encoded by the major open reading frame, encoding the putative RNA-dependent RNA replicase, is shown, as are those of the small open reading frames at the 3' end, corresponding to the proteins P11a (SEQ ID No: 42), P11b (SEQ ID No: 44) and P14 (SEQ ID No: 46).

Clone E3 was inserted downstream of the SP6 promoter for *in vitro* transcription. As mentioned above, the transcript of this clone can be translated in the wheat germ system to yield the 195 kDa protein observed upon translation of fractionated RNA 1 (SEQ ID No: 39) from the virus. The latter yields more lower molecular weight products, presumably due to being contaminated with nicked and degraded RNA. The products derived from the *in vitro* transcript can therefore be regarded as defining the coding capacity of the complete RNA 1 (SEQ ID No: 39) of HaSV.

vi) Sequence of genome component 2 (see Figure 2)

The 2470 nucleotides encode a protein of molecular weight 71,000 which contains the peptide sequences corresponding to those determined from the two virus capsid proteins. This protein is therefore the precursor of these capsid proteins. The protein is a major product of *in vitro* translation of this RNA obtained either from virus particles or by in vitro transcription of a full-length cDNA clone; in addition, another major translation product of apparent molecular weight 24,000 is obtained. This protein is derived from a molecular weight 17,000 reading frame overlappling the slab of the capsid protein gene.

Clones hr236 and hr247 were completely sequenced as the first step in RNA 2 sequencing. These sequences were then extensively compared to that obtained by direct RNA sequencing using AMV reverse transcriptase.

Comparison of the cloned sequence with that by direct RNA sequencing showed both clones lacked 50 nucleotide present in the RNA (at around nucleotide 1500). The sequence of this stretch was obtained by direct RNA sequencing using the AMV RTase. The MMLV "Superscript" RTase, which was used to make all the cDNA clones, was found to simply by-pass this region in sequencing reactions. These 50 nucleotides contain a very stable GC-rich hairpin flanked by a 6 bp direct repeat, and the MMLV RTase skips from the first repeat to the second.

The sequence of RNA 2 (SEQ ID No: 47) was then completed using plasmids pSR2A and pSR2P70 constructed as described below. The plasmids contain a segment of cDNA derived for the AMV RTase, as well as the sequence corresponding to the 5' 240 nucleotides of RNA 2 (SEQ ID No: 47) which are not present on phr236 (Fig. 2). The sequence of RNA in Fig. 2 is presented as the upper strand of the cDNA sequence. This strand is therefore in the same sense as the viral (positive-sense) RNA. The sequences of the proteins encoded by the major open reading frames, encoding the capsid protein precursor P71 (SEQ ID No: 50), and P17 (SEQ ID No: 48).

The sequence of RNA 2 (SEQ ID No: 47) encodes a major ORF running from a methionine initiation codon at nucleotides 366 to 368 to a termination codon at nucleotides 2307 to 2309. This protein encoded by this ORF has a theoretical molecular weight of 71,000 (SEQ ID No: 50). This initiation codon is in a good context (AGGatgG), suggesting that it will be well recognized by scanning ribosomes. The size of the product is close to that of the residual putative precursor protein identified in purified virus, and to the size of the *in vitro* translation product obtained from RNA 2 (SEQ ID No: 47).

The approach adopted to identify the gene encoding the capsid protein was to obtain amino acid sequence information from the two abundant capsid proteins and then locate these on the protein encoded by the sequence of the virus RNA's. CNBr cleaved products of the capsid protein were therefore sequenced. These fragments gave a clear and unambiguous sequence shown in Example 1. These sequences determined were then located on the large ORF of RNA 2 (SEQ ID No: 47). (Figure 2)

In the case of the small capsid protein, the clear and unambiguous sequence, obtained is located near the carboxy terminus of the major ORF on RNA 2 (SEQ ID No: 47). Starting at the point corresponding to the amino-terminal residue of the sequence determined for the 6 kDa protein, and continuing to the carboxy-terminus of the complete reading frame, the protein encoded by the sequence 7.2 kDa and has a hydrophobic N-terminal region and an arginine rich (basic) C-terminal region. It is an extremely basic protein with a pI of 12.6.

The two abundant capsid proteins are derived from a single precursor, which is processed at a specific site. This is presumably immediately amino-terminal to the sequence FAAAVS.... (SEQ ID No: 25)

RNA 2 (SEQ ID No: 47) appears to be a bicistronic mRNA (see Figs. 2 and 5). The first methionine codon is encoded on the sequence of RNA at nucleotides 283 to 285. This ATG is in a poor context (TTTatgA), making it a weaker initiation codon. It initiates a reading frame of 157 amino acids, encoding a protein of molecular weight 17,000 (SEQ ID No: 48). (The second AUG [nts 366 to 368] initiates the 71 kDa (SEQ ID No: 50) precursor of the capsid protein). Since the first AUG is in a poor context, abundant expression of the capsid precursor would be expected. In fact, in vitro translation of a full length RNA 2 (SEQ ID No: 47) transcribed from a reconstructed cDNA clone yields two major protein products of relative mobility 71,000 (SEQ ID No: 50) and 24,000, similar to those already observed upon translation of viral RNA 2 (SEQ ID No: 47). The protein of Mr 24,000 appears to correspond to the 157 amino acid protein, despite the significant anomaly in apparent

size. The 24,000 Mr product was also observed upon translation of an in vitro transcript covering only nucleotides 220 to 1200 of RNA 2 (SEQ ID No: 47). This region contains no open reading frame other than those already mentioned and cannot encode a protein longer than 157 amino acids.

The protein of Mr 24,000 seen upon *in vitro* translation appears to correspond to P17 (SEQ ID No: 48), with the anomaly in apparent size probably being due to the high content of proline (P), glutamate (E), serine (S) and threonine (T). These amino acids cause the protein run more slowly on a gel thereby giving it an apparent size of Mr 24,000.

The Mr 24,000 protein (hereinafter referred to as P17 (SEQ ID No: 48)) may have a function in modifying or manipulating the growth characteristics or cell cycle of HaSV-infected cells. Although a protein of 16kDa (identified in Example 1) is found in small amounts in the capsid, it does not react with antiserum against the virus particles this is unlikely to correspond to P17 (SEQ ID No: 48), since a preparation of the latter proteins migrates with a molecular weight of 24,000 on SDS gels.

Sequence analysis of the Region from nucleotide 500 to 600 of RNA 2 (SEQ ID No: 47) showed that it has the sequence shown in Fig. 2, as do the plasmids pSR2A, pSR2P70, pSR2B and pSXR2P70. However, plasmids pT7T72P65 and pT7T2P70 have an extra C residue at nucleotide 570. The RNA sequence from which they are derived from is shown in Fig. 2 (the "5C" version). In this sequence the first ATG (nucleotides 283 to 285) is in the same reading frame as most of the capsid protein gene. The resultant fusion protein is called "P70" (SEQ ID No: 52) and its carboxyterminal-truncated version (a variant of the native P64) is "P65". In view of these clones it was considered important to resolve whether any virus RNA carrying the extra C residue was present in the viral RNA population first isolated for investigation.

Direct sequencing of the virus RNA using reverse transcriptase confirmed that the 4C version lacking the extra residue was the abundant form of the RNA. In order to exclude the possibility of a small amount of the RNA having the extra residue, a sensitive PCR assay was designed. This showed that the extra C residue was not present on any RNA in the viral population, and had been introduced into some clones as a PCR artefact. These clones were however retained and used in bacterial expression experiments (below) because of the high level expression obtained of the P65 and P70 (SEQ ID No: 52) fusion proteins.

vii) Comparison with the sequence of the *Nudaurelia* w capsid gene

The sequence of most of the RNA2 of the *Nudaurelia* w virus has recently been published by Agrawal D.K. and Johnson J.E. (Virology 190 806-814, 1992). From the published sequence it has been determined that this sequence shows 63% homology to that of HaSV RNA2 (SEQ ID No: 47) at the nucleotide level and 66% at the overall amino acid level. A detailed comparison of the capsid proteins of these two viruses shows the amino-terminal 45 residues to be variable, the next 220 residues to be highly conserved, the next 180 residues to be variable and the c-terminal 200 residues covering the small protein P7 to be highly conserved. A more detailed comparison is discussed below.

The published report did not find a complete reading frame corresponding to the 157 amino acid protein (P17 (SEQ ID No: 48)) gene reported above. The AUG is however present, as is a reading frame - starting upstream of the start of the capsid gene - showing considerable amino acid homology to P17 (SEQ ID No: 48) of HaSV. In vitro translation of purified *Nudaurelia* w virus RNA 2 and a re-examination of the nucleotide sequencing data for this RNA may help to resolve the question of whether the *Nudaurelia* w virus also encodes a protein homologous to the HaSV P17.

More interestingly, antisera against these two viruses, which are similar at a nucleotide sequence level, do not show any cross-reactivity.

viii) Construction of full-length clones

RNA 1 (SEQ ID No: 39)

cDNA clone E3, described above contains all but the 5'-18 nucleotides of RNA 1 (SEQ ID No: 39) and included the complete ORF present on the sequence. The first full-length clone of RNA 1 (SEQ ID No: 39) is therefore based on E3. The 4.9 kbp XbaI-ClaI fragment from clone E3 was recloned into pBSKS(-) (Stratagene) cut with XbaI and ClaI, giving pBSKSE3.

The full-length clone of RNA 1 (SEQ ID No: 39) was completed using PCR. The primer defining the 5' end of the RNA carried an EcoRI site, the promoter for the SP6 RNA polymerase and a sequence corresponding to the 5' 17 nucleotides of RNA 1, as shown in Figure 1. The sequence of this primer was:

HvR1SP5p:

5'-GGGGGGAATTCATTTAGGTGACACTATAGTTCTGCCTCCCCGGAC (SEQ ID No: 11) (The G which initiates transcription is underlined)

Using an oligonucleotide complementary to nucleotides 1192 - 1212, a PCR product of 1240 bp was efficiently made. The template was cDNA synthesised using the MMLV RTase and the same oligonucleotide complementary to nucleotides 1192 - 1212 was the primer. Upon termination of the PCR reaction, the product's ends were made blunt and then 5'-phophorylated as described below. The purified PCR fragment was then cleaved with restriction endonuclease XbaI and the 450 bp subfragment corresponding to the 5' end of RNA 1 (SEQ ID No: 39) cloned into the plasmid pBSSK(-)(Stragene) cut with EcoRV and XbaI, to give pBSR15.

To assemble the full-length of RNA 1 (SEQ ID No: 39), pBSKSE3 (above) was cut with XbaI and ScaI giving fragments of 1.2 kbp and 6.8 kbp. pBSR15 was cut with the same enzymes, giving fragments of 2 and 1.8 kbp. Ligation of the 6.8 kbp fragment for pBSKSE3 and the 1.8 kbp fragment for mpBSR15 yielded pSR1(E3)A. Upon linearization at ClaI and *in vitro* transcription with the SP6 RNA polymerase,

and RNA corresponding to RNA 1 (SEQ ID No: 39), and terminating in a poly(A) stretch of about 50 nucleotides, is obtained.

Since the natural RNA 1 (SEQ ID No: 39) does not have a poly (A) tail, an alternative plasmid was constructed which carries a BamHI restriction site immediately downstream of the 3'end of RNA 1 (SEQ ID No: 39). Again this terminal fragment was made using PCR as above. The sequence of the primer was as follows: HvR13p: 5'-GGGGGGATCCTGGTATCCCAGGGGCGC (SEQ ID No: 12) (the nucleotide complementary to that which was determined as the 3' one, based on its adjacency to the poly(A) stretch, is underlined; RNA terminating at the BamHI site will have the sequence GCGCCCCCUGGGAUACCaggauc (SEQ ID No: 26)).

The template was clone E3 and an oligonucleotide corresponding to nucleotides 4084 - 4100 was the other primer. The 1220 bp product was blunt-ended, kinased and gel-purified as described above, before cleavage with HindIII. The resulting 420 bp subfragment corresponding to the 3' end of RNA 1 (SEQ ID No: 39) cloned into plasmid pSR1(E3)A cut with ClaI, end-filled with Klenow and then cut with HindIII. The resulting plasmid is pSR1(E3)B. Upon linearization at BamHI and *in vitro* transcription with the SP6 RNA polymerase, and RNA corresponding to RNA 1 (SEQ ID No: 39), and terminating as described immediately above is obtained.

ix) RNA 2 (SEQ ID No: 47)

In constructing the full-length cDNA clone to enable *in vitro* transcription of this RNA hr236 described above was used as a basis. Two separate PCR products, one corresponding to the 5' portion of RNA 2 (SEQ ID No: 47), which is missing from this clone altogether, and another covering the region where clone hr236 lacks the hairpin-forming sequence described above, were required.

The primer defining the 5' end of the RNA carried a HindIII site and a sequence corresponding to the 5' 18 nucleotides of RNA 2 (SEQ ID No: 47), as shown in Figure 2. The sequence of this primer was:

Hr2cdna5: 5'-CCGGAAGCTTGTTTTTCTTTCTTTACCA (SEQ ID No: 13)
(The nucleotide underlined corresponds to that identified as the first nucleotide of RNA 2. (SEQ ID No: 47))

Using an oligonucleotide complementary to nucleotides 1653 - 1669, a PCR product of 1.67 kbp was made. The template was cDNA synthesised using the MMLV RTase and an oligonucleotide complementary to the 18 nucleotides at the 3' end of RNA 2 (SEQ ID No: 47) as the primer. Upon termination of the PCR reaction, the product was blunt-ended, kinased and gel-purified as described above, before cleavage with PstI. The resulting 1.3 kbp subfragment corresponding to the 5' half of RNA 2 (SEQ ID No: 47) was cloned into plasmid pBSSK(-) (Stragene) cut with EcoRV and PstI, giving plasmid pBSR25p. In order to place this subfragment corresponding to the 5' half of RNA 2 (SEQ ID No: 47) downstream of the SP6 promoter for *in vitro* transcription, a 1.3 kbp HindIII - BamHI fragment was excised from pBSR25p and ligated into HindIII - BamHI cut pGEM-1 (Promega), giving plasmid pSR25.

The second PCR product, covering the region where clone hr236 lacks the hairpin-forming sequence described above, was synthesised using as primers oligonucleotides corresponding to nucleotide sequence 873 to 889 of RNA 2 (SEQ ID No: 47) and to the complement of nucleotide sequence 2290 - 2309. Upon termination of the PCR reaction, the product was blunt-ended, kinased and gel-purified as described above, before cleavage with AatII. The resulting 1.1 kbp subfragment covering the required region was cloned into plasmid phr236 cut with HindIII, end-filled with Klenow and cut with AatII, giving plasmid phr236P70.

The two segments were joined covering the first 230 nucleotides of RNA 2 (SEQ ID No: 47) together. Plasmid phr236P70 was cut at the SacI site in the vector adjacent to the 5' end of the insert and this made blunt-ended using Klenow in the absence of dNTPs. After heat-inactivation of the Klenow, the plasmid was cut with EcoRI, yielding fragments of 4.5 kbp and 380 bp. Plasmid pSR25 was cut with NheI, blunt-ended by end-filling with Klenow and cut with EcoRI, yielding fragments of 2.8 kbp, 900 bp and 750 bp. The 4.5 kbp fragment of phr236P70 and the 900 bp fragment of

pSR25 were ligated to give pSR2P70. This clone covers all of RNA 2 (SEQ ID No: 47) except for the 3' 169 nucleotides.

To complete the full-length clone of RNA 2 (SEQ ID No: 47), it was necessary to insert a fragment covering the 3' end. As with RNA 1 (SEQ ID No: 39), two versions were made. One, called pSR2A, used the 3' end as present in phr236, together with the poly(A) tail present in this version. The other pSR2B, used a PCR fragment carrying a BamHI site immediately downstream of the 3' nucleotide, as in pSR1(E3)B above. To construct pSR2A, a 350 bp NotI-ClaI fragment was excised from phr236 and cloned into pSR2P70 cut with the same endonucleases. Linearization at the unique ClaI site allows *in vitro* transcription of the complete RNA 2 (SEQ ID No: 47) and a poly(A) tail of about 50 nucleotides in length.

To make pSR2B, an appropriate PCR product was made using as primers an oligonucleotide corresponding to nucleotide sequence 1178 to 1194 and to the 3' terminal 18 nucleotides of RNA 2 (SEQ ID No: 47). The latter primer carried a BamHII site attached, giving it the sequence:
HvR23p: 5'-GGGGGATCCGATGGTATCCCGAGGGACGC
(SEQ ID No: 14)

The template used was a plasmid phr236. Upon termination of the PCR reaction, the product was blunt-ended, kinased and gel-purified as described above, before cleavage with NotI. The resulting 400 bp subfragment covering the required region was cloned into plasmid pSR2P70 cut with ClaI, end-filled with Klenow and cut with NotI, giving plasmid pSRP2B. Linearization at the unique BamHI site allows *in vitro* transcription of the complete RNA 2 (SEQ ID No: 47), terminating with the sequence ACCaggatc.

x) Construction of pSXR2P70

This plasmid was made to determine where p24 starts. A 2.1 kbp XhoI-BamHI fragment was cut from clone pSR2P70 and ligated into the vector pGEM-1 (Promega) which had been cut with SalI and BamHI. In vitro transcription of the resulting

plasmid after linearization at the unique BamHI site yielded an RNA covering about 70 nucleotides upstream of the first ATG at nucleotides 283 to 286, plus a short sequence derived from the vector.

In vitro translation of the RNA from pSXR2P70 yielded both proteins (P70 (SEQ ID No: 52) + P24).

xi) Description of virus-induced pathology

The virus induces a rapid anti-feeding effect in Helicoverpa larvae as determined by experiments with larvae the results of which are shown in Fig. 3. Fig. 3 shows: A. neonate larvae (less than 24 h old) were fed the designated concentrations of isolated virus (in particles per ml [of diet] added to solid diet). They were weighed on following days and the mean of a statistically significant number (24) of larvae shown. Where necessary, mortality was recorded for the higher concentrations. The vertical axis shows the fold-increase in weight from the hatching weight of 0.1 mg per larvae. This scale therefore also corresponds to weight in units of 0.1 mg (ie 300 is equivalent to 30 mg). B. As for A, but the larvae were 5 days old at the start of the virus feeding. The vertical scale is in mg weight.

No weight gain at all was detectable with neonates which had been fed the doses of virus over 10^8 particles per ml (virus added to diet). In addition, 100% mortality was evident after four days at the highest doses. Virus doses as low as 10^6 particles per ml (virus added to diet) still cause significant stunting. The five day old larvae showed a cessation of feeding after 48 hours and significant stunting at 4 dpi, but no mortality at comparable virus doses (Figure 3). Neonates are therefore very sensitive indeed to this virus. Virus particles accumulate specifically in the midgut. This potent anti-feeding effect may be due to the capsid protein or another protein encoded by the virus, or to the effect of any combination of such proteins.

xii) Expression of virus-encoded proteins in bacteria.

The vectors

The expression system used initially was derived from the pET-11 system (Novagen). Trimmed down versions of pET-11b and c were constructed and used to compare expression of the capsid proteins. However, due to difficulties experienced with this system substantial modification of the original vectors was carried out in order to achieve much higher yields. These results are described in xiii-b) below.

The initial trimmed-down vectors discussed above were made as follows: pGEM-2 (Promega) which carries T7 promoter adjacent to a poly-linker sequence, but has no sequences corresponding to the lac operon, was cut at the unique XbaI (34) and ScaI (1651) sites, giving fragments of 1.61 and 1.25 kbp. The plasmids pET-11b and c were cut with the same enzymes, giving fragments of 4.77 and 0.91 kbp. The 1.61 kbp fragment of pGEM-2, carrying the c-terminal portion of the ampicillin-resistance gene, the origin of replication and the T7 promoter, was then ligated to the 0.91 kbp fragment of the pET vector, which carries a sequence covering the Shine-Dalgarno sequence, the ATG (in a NdeI site), the terminator for the T7 polymerase and the N-terminal portion of the ampicillin-resistance gene. The resulting plasmids of approximately 2.53 kbp, called pT7T2-b and c, therefore carry a complete T7 transcription unit, which may be used as an expression system in a manner similar to the original pET-11 plasmids, but are repressor-neutral within the cell; they neither titrate away repressor by carrying a binding site, nor do they carry the gene producing the repressor. They were found to grow very well in *E.coli* strains JM109 and BL21 (DE3), and to be very efficient expression vectors. The repressor present in the cells was found to be sufficient to keep the genomic T7 polymerase gene uninduced and therefore the foreign gene unexpressed in the absence of IPTG.

xiii-a) Construction of plasmids for expression of capsid proteins

In this section, all proteins expressed from segments of HaSV RNA 2 (SEQ ID No: 47) are referred to by the size of their gene, as defined in Fig. 4 and in section vi) of this example. The following plasmids were constructed by PCR, using the abovementioned full-length clone of RNA 2 (SEQ ID No: 47), plasmid pSR2A as the template, except where mentioned otherwise.

Groups of plasmids expressed protein starting at each of the first three methionine initiation codons found on the sequence of HaSV RNA 2 (SEQ ID No: 47). For those proteins initiating at the first methionine initiation codon found on the sequence of HaSV RNA 2 (SEQ ID No: 47) (which initiates the P17 (SEQ ID No: 48) gene; oligonucleotide primer HVPET65N (SEQ ID No: 15)), an extra group of plasmids was made by PCR using as a template the version of the RNA 2 sequence carrying an extra C residue inserted at residue 570 (SEQ ID No: 51) (as depicted in Figure 2). Expression constructs initiating at the third methionine initiation codon found on the sequence of HaSV RNA 2 (which is located within the P17 gene; oligonucleotide primer HVPET63N (SEQ ID No: 16)) were made by PCR using as a template only the version of the RNA 2 sequence carrying an extra C residue inserted at residue 570 (SEQ ID No: 51). For these latter expression constructs, as well as those designed to initiate expression from the second methionine initiation codon found on the sequence of HaSV RNA 2 (SEQ ID No: 47) (which initiates the P71 gene; oligonucleotide primer HVPET64N (SEQ ID No: 17)), two versions were constructed.

One version terminated at a point corresponding to the c-terminus of the processed (P64) form of the capsid protein and was made using oligonucleotide primer HVP65C (SEQ ID No: 19). The other version terminated at a point corresponding to the c-terminus of the precursor (P71 (SEQ ID No: 50)) form of the capsid protein and was made using oligonucleotide primer HVP6C2 (SEQ ID No: 20).

The sequence encoding P64 (or the precursor, P71 (SEQ ID No: 50)) was synthesised in two segments using PCR. The amino-terminal half of the gene was obtained using as primers oligonucleotides incorporating one of the three ATG possible initiation codons for the ORF, in addition to an oligonucleotide with the sequence TCAGCAGGTGGCATAGG (SEQ ID No: 27); complementary to nucleotides 1653 to 1669 of the sequence shown in Fig. 2. The forward primers were as follows:

HVPET65N:

AAATAATTTTGTTTACTTTAGAAGGAGATATACATATGAGCGAGCGAGCAC AC (SEQ ID No: 15)

(the underlined sequence corresponds to nucleotides 283 to 296 of the sequence shown in Figure 2)

HVPET63N

AAATAATTTTGTTTAAC**CTT***A***AG**AAGG**AGAT***C***T**ACAT<u>ATGCTGGAGTGGCGTCAC</u> (SEQ ID No: 16)

(the underlined sequence corresponds to nucleotides 373 to 390 of the sequence shown in Figure 2; the AflII (CTTAAG) and BglII (AGATCT) sites introduced into the sequence by single nucleotide changes (shown in *italics*) in the oligonucleotide are shown in **bold**).

HVPET64N

GGAGATCTACATATGGGAGATGCTGGAGTG (SEQ ID No: 17)

(the underlined sequence corresponds to nucleotides 366 to 383 of the sequence shown in Figure 2; the BglII site introduced into the sequence by a single nucleotide change in the oligonucleotide is shown in **bold**).

The PCR products obtained from each combination of one of these primers with the abovementioned one were treated with the Klenow fragment of *E.coli* DNA polymerase, and then with T4 polynucleotide kinase in the presence of 1 mM ATP, before purification by agarose gel electrophoresis as described above. Each product was then cleaved with AatII to yield fragments of 0.95 and 0.4 kbp, and each resulting fragment of about .95 kbp cloned intro vector pGEM-2 (Promega) cut with HincII and AatII, giving plasmids pGEMP63N (in which the insert commenced with oligonucleotide HVPET63N (SEQ ID No: 16)), pGEMP64N (in which the insert commenced with oligonucleotide HVPET64N (SEQ ID No: 17)) and pGemP65N (in which the insert commenced with oligonucleotide HVPET65N (SEQ ID No: 15)). The fragment covering portion of the HaSV capsid gene was then excised with enzymes AatII and XbaI.

Two versions of plasmid pGemP65N were made, using different templates as described above. pGemP65N was derived from the sequence of the viral RNA, as in plasmid pSF2A; plasmid pGemP65Nc was derived from the sequence carrying an extra C residue, as shown in Fig. 2 (see "5C version").

In parallel, the carboxy-terminal halves of the major capsid protein variant, whether terminating as for P64 or for P71 (SEQ ID No: 50), were also produced using PCR. An oligonucleotide primer, HVRNA2F3, with the sequence GTAGCGAACGTCGAGAA (SEQ ID No: 18) (corresponding to nucleotides 873 to 889 of the sequence shown in Figure 2) was used in conjunction with each of the two primers following:

HVP65C
GGGGGATCCTCAGTTGTCAGTGGCGGGGTAG (SEQ ID No: 19)
(the underlined sequence is complementary to nucleotides 2072 to 2091 of the sequence shown in Figure 2).

HVP6C2
GGGGATCCCTAATTGGCACGAGCGGCGC (SEQ ID No: 20)
(the underlined sequence is complementary to nucleotides 2290 to 2309 of the sequence shown in Figure 2).

The PCR products obtained from each combination of one of these primers with the above mentioned one (HvRNA2F3 (SEQ ID No: 18)) were treated with the Klenow fragment of *E.coli* DNA polymerase, and then with T4 polynucleotide kinase in the presence of 1 mM ATP, before purification by agarose gel electrophoresis as described above. Each product was then cleaved with AatII to yield fragments of 0.9 kbp (in the case of HVP65C (SEQ ID No: 19)) or 1.1 kbp (in the case of HVP6C2 (SEQ ID No: 20)) and 0.4 kbp, and each resulting fragment of about .9 or 1.1 kbp cloned into plasmid phr236 cut with HindIII, treated with Klenow and AatII, giving plasmids phr236P65C and phr236P70 (which has already been described above), respectively. The fragment covering the c-terminus of the capsid protein gene was then excised with enzymes AatII and BamHI.

To assemble plasmids for expression in suitable strains of *E. coli*, the excised XbaI-AatII fragments of 0.95 kbp covering the amino-terminal half of the gene and the

excised AatII - BamH1 fragments of 0.9 or 1.1 kbp covering the carboxy-terminal half of the gene were simultaneously ligated into the vector pT7T2 cut with XbaI and BamHI. Initial transformation was of *E. coli* strain JM109. Recombinant plasmids carrying the correct insert were then transformed into strain BL21(DE3) for expression as described above.

The plasmid obtained by ligating the aminoterminal fragment commencing with oligonucleotide primer HVPET63N (SEQ ID No: 16) to the c-terminal fragment ending at oligonucleotide primer HVP65C (SEQ ID No: 19) in the epxression vector pT7T2b was called pP65G.

In the case of plasmid pP64N, containing an insert from HVPET64N (SEQ ID No: 17) to HVP65C (SEQ ID No: 19), the fragment covering the amino-terminal half of the oligonucleotide was excised by BglII and ScaI from the plasmid pGemP64N and the fragment covering the remainder of the gene was excised with ScaI and EcoRI from plasmid pT7T2-P65. These two fragments were then ligated simultaneously into pP65G which had been cut with BglII sand EcoRI.

The resulting construct carrying the complete P71 (SEQ ID No: 50) precursor gene was called pT7T2-P71 and that carrying the P64 form of the gen was called pT7T2-P64. In the case of plasmids derived from pGemP65N and pGemP65Nc, carrying inserts commencing as defined by primer HVPET65N, the expression plasmid derived from pGemP65N which is based on PCR products made using as the template the sequence of the viral RNA, as in plasmid pSR2A, was called pTP17; a truncated form of this plasmid, which expresses P17 (SEQ ID No: 48), was made by cutting at the unique BglII and BamHI sites, removing the intervening fragment (which corresponds to the c-terminal part of the insert) and religating the compatible cohesive ends, to give pTP17delBB. The expression plasmids derived from plasmid pGemP65Nc (which was derived from the sequence carrying an extra C residue, were called pT7T2-P65 (carrying an insert terminating at the primer HVP65C (SEQ ID No: 19)) and pT7T2-P70 (carrying an insert terminating at the primer HVP6C2 (SEQ ID No: 20)).

Expression of P6

Two forms of this protein, which arises through processing of the large capsid protein variant precursor P70 (SEQ ID No: 52) and therefore lacks its own initiation codon, were made. One form (protein MA) replaced the phenylalanine at the start of this protein with methionine, giving it the amino-terminal sequence MAA...; the other carries an additional methionine residue, giving it the amino-terminal sequence MFAA... The oligonucleotides used for PCR-amplified products covering the p6 coding sequence carried a NdeI site (bold) at the ATG codon, for direct ligation into the pET-1I vectors. The primers used were:

HVP6MA: AATTACATATGGCGGCCGCCGTTTCTGCC (SEQ ID No: 21)

HVP6MF: AATTACATATGTTCGCGGCCGCCGTTTCT (SEQ ID No: 22)

Each of these primers was used in conjunction with primer HVP6C2 (SEQ ID No: 20) to generate a PCR product of 0.2 kbp. These products were blunt-end ligated into vector pBSSK(-) which had been cut with EcoRV and dephosphorylated. The insert corresponding to the p6 gene was excised with NdeI and BamHI (using the BamHI site in the primer HVP6C2 (SEQ ID No: 20)) and ligated into the expression vector pET-1Ib, which had been cut with the same enzymes. For expression at higher levels, the insert was transferred to PT7T2 as a XbaI - BamHI fragment, yielding plasmids pTP6MA and pTP6MF.

IPTG induction of bacteria containing plasmids pTP6MA or pTP6MF were used produce p6 for bioassay.

xiii-b) Expression of viral genes in *E. coli* and bioassay in larvae

Expression of P64

IPTG induction of bacteria containing plasmid pT7T2-P65, which contains an insert running from the location of primer HVPET65N (SEQ ID No: 15) to that of primer HVP65C (SEQ ID No: 19), yielded a protein of molecular weight 68 000. This was 3

000 molecular weight greater than the size of the authentic coat protein, as expected. Expression of pP65G, which contains an insert running from HVPET63N (SEQ ID No: 16) to HVP65C (SEQ ID No: 19), yielded a protein of 65 000 molecular weight.

The authentic capsid protein (P64) was expressed poorly from plasmid pT7T2-P64. Recloning this insert as a NdeI-BamHI fragment back into the other form of the vector (PT7T2b) did not alter this.

Expression of P70

IPTG induction of bacteria containing plasmid pT7T2-P70, which contains an insert running from the location of primer HVPET65N (SEQ ID No: 15) to that of primer HVP6C2 (SEQ ID No: 20), yielded a protein of molecular weight 73 000. This was 3 000 molecular weight larger than the size of the precursor of the coat protein, as expected.

The authentic capsid protein precursor (P71 (SEQ ID No: 50)) was expressed poorly from plasmid pT7T2-P71. Recloning this insert as a Ndel-BamHI fragment back into the other form of the vector (pT7T2b) did not alter this.

Due to the observation mentioned in vi) above, plasmids designed to express all forms of the capsid proteins from several possible ATG's at the start of the open reading frame were constructed.

It was found that both authentic P64 and P71 (SEQ ID No: 50) were expressed poorly in bacteria. In contrast, P17 (SEQ ID No: 48) and the forms of the capsid protein commencing at the P17 ATG were expressed very well. The extra C residue present in the latter two constructs resulted in a fusion protein being made from these expression plasmid. The sequence of the fusion proteins can be derived from Fig. 2 by including an extra C at position 570. The fusion caused the first 67 residues of the HaSV capsid protein to be replaced by the first 95 residues of P17 (SEQ ID No: 48). Good expression of the large capsid precursor and protein was achieved, but the size of these

proteins were above 3 kDa larger than the authentic forms. Notwithstanding this the expression products of the vectors containing the 5C variant of RNA 2 (SEQ ID No: 51) are still useful because the resulting product, a P70 (SEQ ID No: 52) variant, is only modified at the NH_2 terminus. Since this terminus is thought to be embedded in the capsid structure and therefore not to participate in the initial interaction with the larval midgut cell, the variant is still useful.

In order to produce constructs which ensure that the expressed proteins possessed the native amino terminus, new plasmids carrying the correct sequence were then cloned into the expression vector (pT7T2). It was found these plasmids to express proteins of the correct size.

The P6 has not yet been to expressed from the new constructs. No evidence has been found for processing of P70 to yield the mature proteins in bacteria, nor upon *in vitro* translation of synthetic full-length RNA 2 (SEQ ID No: 47).

The P17 (SEQ ID No: 48) gene has also been cloned into the same vectors for expression and bio-assay. This protein accumulates well in bacteria upon induction, and electron microscopy analysis has shown it form spectacular honeycomb-like structures under the bacterial cell wall, completely surrounding the cell interior (results not shown). The properties of this protein including its amino acid composition and ability to form tube-like structures when expressed in bacteria suggest that it may be an homolog of a gap junction protein. The latter is involved in forming the channels linking the cytoplasms of adjacent epithelial cells in the insect gut. P17 could then play a role in enlarging or forming these channels, thereby enabling cell-to-cell movement of the virus in the insect gut, analogous to the movement or spreading proteins encoded by plant RNA viruses.

In order to ensure that the expressed proteins carried the native amino terminus the correct sequence has also been cloned into the expression vector (pT7T2). The vector had been very slightly modified to that described above to introduce two novel

restriction sited (for AflII and BgIII) flanking the Shine-Dalgarno sequence. The resulting constructs have been found to be poor producers of the capsid proteins. The complete coding regions (which have been completely checked by re-sequencing) have therefore been recloned into the more satisfactory vectors. Results using these constructs suggest that the amino-terminus of the capsid protein presents inherent difficulties in expression. These difficulties may be imposed by either the nucleotide sequence encoding the amino terminus, or the actual amino acid sequence itself. To discriminate between these possibilities, two types of mutants were made in the sequence encoding the amino terminal 5 residues of the HaSV capsid protein. These amino-terminal mutants are as follows:

HVP71GLY

CCCATATG GGC GAT GCC GGC GTC GCG TCA CAG (SEQ ID No: 28)

Met Gly Asp Ala Gly Val Ala Ser Gln (SEQ ID No: 29)

HVP71SER:

CCCATATG AGC GAG GCC GGC GTC GCG TCA CAG (SEQ ID No: 30)

Met Ser Glu Ala Gly Val Ala Ser Gln (SEQ ID No: 31)

Native HaSV seq:

ATG GGA GAT GCT GGA GTG GCG TCA CAG (SEQ ID No: 32)

Met Gly Asp Ala Gly Val Ala Ser Gln (SEQ ID No: 33)

EXAMPLE 4

EXPRESSION IN BACULOVIRUS VECTORS AND BIOASSAY ON LARVAE

Materials and Methods

A(i) Cloning of HaSV capsid protein gene.

The capsid protein gene was amplified by PCR using the following primers:

5' primers:

HV17V71:

5' GGGGGATCCCGCGGATTTATGAGCGAG (SEQ ID No: 34)

HV17E71:

5' GGGGGATCCCGCGGAGACATGAGCGAGCACAC (SEQ ID No: 35)

HVP71:

5' GGGGGATCCAGCGACATGAGAGATGCTGGAGTGG

(SEQ ID No: 36)

HVV71:

5' GGGGGATCCAGCGACATGAGAGATGCTGGAGTGG

(SEQ ID No: 37)

The ATG triplets initiating P17 (SEQ ID No: 48) (in HV17V71 (SEQ ID No: 34) and HV17E71 (SEQ ID No: 35)) or P71 (SEQ ID No: 50) (in HVP71 and HVV71) are underlined)

3' primers:

Primers HVP65C (SEQ ID No: 19) and HVP6C2 (SEQ ID No: 20), described in Example 3. Results section Xiiia, were used. These constructs were made using one of the four 5' primers and HVP6C2 (SEQ ID No: 20). Plasmids constructed from PCR products made using one of the four 5'- primers and HVP65C (SEQ ID No: 19) are called 17V64 (made using 5' primer 17E71 (SEQ ID No: 35)), P64 (made using 5' primer P71 (SEQ ID No: 36)) and V64 (made using 5' primer V71 (SEQ ID No: 37)). These plasmids allow expression of P64.

A(ii) Cloning a full length cDNA of HaSV RNA 1 (SEQ ID No: 39).

For expression of an RNA transcript corresponding to full length HaSV RNA 1 (SEQ ID No: 39), in insect cells by baculovirus infection or plasmid transfection, PCR was used to generate a fragment of cDNA linking the 5' end of RNA 1 (SEQ ID No: 39) to a Bam HI site.

The primers were:

HVR1B5'

5' GGGGGATCCGTTCTGCCTCCCCGGAC (SEQ ID No: 38)
(where the underlined nucleotide represents the start of natural RNA 1 (SEQ ID No: 39)), and an oligonucleotide complementary to nucleotides 1192=1212 of RNA 1 (SEQ ID No: 39).

The template was plasmid pSR1(E3)B described in Example 3 above.

A segment of the 1240 bp PCR fragment corresponding to the 5' 320 nucleotides of RNA 1 (SEQ ID No: 39) was excised with Bam HI and ASC II and cloned into the Bam HI site of pBSSK(-)[Stratagene] together with the 5 kbp ASCII - Bam HI fragment of pSR1(E3)B, giving plasmid pBHVR1B, which carries the complete cDNA to HaSV RNA 1 (SEQ ID No: 39), flanked by Bam HI sites.

A(iii) Cloning a full length CDNA of HaSV RNA 2 (SEQ ID No: 47).

For expression of an RNA transcript corresponding to full length RNA 2 (SEQ ID No: 47) in insect cells by baculovirus infection or plasmid transfection, plasmid pB+NR2B was made by inserting a fragment carrying Hind III and Bam HI sites from the multiple cloning site of vector pBSSK(-) [Stratagene] into plasmid pSR2B described above. The resulting plasmid, called pBHVR2B, carried the cDNA corresponding to full length HaSV RNA 2 (SEQ ID No: 47), flanked by Bam HI sites.

A(iv) Baculovirus transfer plasmids.

Bam HI fragments of 5.3 and 2.5 kbp corresponding to HaSV RNA's 1 and 2 (SEQ ID Nos: 39 and 47) respectively, were excised from pBHVR1B and pBHVR2B respectively and inserted into the baculovirus transfer vectors described below, which had been linearised with Bam HI.

B. Baculovirus Expression of Proteins.

Baculovirus transfer vectors and engineered AcMNPV virus were transfected into *Spodoptera frugiperda* (SF9) cells as described by the supplier (Clontech) and as described in the following references:

Vlak, J.M. & Kens, R.J.A. (1990) in 'Viral Vaccines", Wiley-Liss Inc., NY, pp.92-128; Kitts, P.A. et al (1990) Nucleic Acids Research 18: 5667-5672; Kitts, P.A. and Possee, R.P. (in preparation); Possee, R.D. (1986) Virus Research, 5: 43-59.

C. Western Blotting.

As in Example 1

D. Oligonucleotides.

The following Ribozyme Oligonucleotides were produced according to standard methods.

HVR1Cla

5' CCATCGATGCCGGACTGGTATCCCAGGGGG (SEQ ID No: 5)

5' HVR2Cla

5' CCATCGATGCCGGACTGGTATCCCGAGGGAC (SEQ ID No: 6)

RZHDV1

5' CCATCGATGATCCAGCCTCCTCGCGGCGCCGGATGGGCA (SEQ ID No: 7)

RZHDV2

5' GCTCTAGATCCATTCGCCATCCGAAGATGCCCATCCGGC (SEQ ID No: 8)

RZHC1

5' CCATCGATTTATGCCGAGAAGGTAACCAGAGAAACACAC (SEQ ID No: 9)

RZHC2

5' GCTCTAGACCAGGTAATATACCACAACGTGTGTTTCTCT (SEQ ID No: 10)

Results

A series of recombinant baculoviruses has been constructed, based on the pVL941 transfer vector (PharMingen) or pBakPak8 (Clontech) and the AcMNPV. These are

designed to express the correct forms of the precursor and processed HaSV capsid proteins (P64 and P71 (SEQ ID No: 50)) as well as the smaller capsid protein P6, and P17 (SEQ ID No: 48). In all systems where replicatable RNA encoding the nucleotide sequences of the present invention are to be used, such as eukaryotic systems, in order to get efficient replication, translation or encapsidation of the RNA it is necessary to excise structures downstream of the t-RNA like structure such as the 3' extension or poly A tail on the RNA. In order to carry out such an excision, ribozymes or other suitable mechanisms may be employed. This self cleavage activity of the ribozyme containing transcript should proceed at such a rate that most of the transcript is transported into the cytoplasm of the cell before the regeneration of a replicatable 3' end occurs. Such ribozyme systems are more fully explained in Examples 7 and 9. In the results presented here highly efficient production of P64 and P71 (SEQ ID No: 50) has been achieved. Electron microscopy and density gradient analysis have confirmed that empty particles ("capsoids") are being produced in infected cells that efficiently express the P71 precursor gene. P17 (SEQ ID No: 48) placed in the context of the *H. virescens* juvenile hormone esterase (JHE) gene (Hanzlik T.N., et al, J. Biol. Chem. 264, 12419-25 (1989)) is produced, but not in large amounts. The latter construct results in a reduction of expression of the capsid protein from the same recombinant, presumably due to a reduction in the number of ribosomes reaching the AUG for the capsid gene.

SF9 cells infected with recombinant baculovirus have been shown to contain large amounts of icosahedral virus particles by electron microscopy (data not shown). These particles contained no RNA, and were empty inside. This observation shows that signals on the viral RNA required for encapsidation of RNA must be located in either the 5' 270 nucleotides or the 3' 170 nucleotides, or both, since these sequences were missing from the RNA transcripts made using recombinant baculovirus. Expression of HaSV proteins was confirmed by Western blotting of total protein extracts from infected insect cells.

In addition, the pAcUW31 vector (Clontech), which carries two promoters, is being used to simultaneously express p6 and p64 as separate proteins.

In order to bioassay the capsid protein produced in baculovirus infected cells, it is first necessary to purify it from the baculovirus expression vector. Preliminary attempts have made use of density gradients, based on the observation that empty virus particles ("assembled capsids") are in fact produced in infected cells.

As outlined earlier, the HaSV genome or portion thereof is a particularly effective insecticidal agent for insertion into baculovirus vectors. Such a vector is constructed by insertion of the complete virus genome or portion thereof (preferably the replicase gene) into the baculovirus genome as shown in Fig. 13. Preferably the virus genome or replicase is transcribed from a promoter active constitutively in insect cells or active at early stages upon baculovirus infection. An example of such a promoter is the heat shock promoter described in Example 7. Heat shock promoters are also activated in stressed cells, for example cells stressed by baculovirus infection. An even more preferable use of such a baculovirus construct is to use the HSP promoter to drive the HaSV replicase and another gene for a toxin (as exemplified elsewhere in the specification) where the RNA expressing the toxin gene is capable of being replicated by the HaSV replicase. Such recombinant baculoviruses carrying the HaSV genome or portions thereof for expression in larvae at early or other stages of the baculovirus infection cycle are particularly effective biological insecticides.

EXAMPLE 5

EFFECT OF HaSV GENES AND THEIR PRODUCTS ON PLANTS

Materials and Methods

A. Electroporation of protoplasts.

Protoplasts of *Nicotiana tobacum, N. plumbaginifolia and Triticum aesticum* and oats were produced and electroporated with either HaSV or HaSV RNA as described in Matsunaga et al (1992) J.Gen. Virol. 73: 763-766.

B. Northern blot analysis - RNA extraction from protoplasts after harvest

The protoplasts are subjected to 3 cycles of freezing and thawing, and then an equal volume of 2x extraction buffer (100 mM Tris-HCl, pH 7.5, 25 mM EDTA, 1% SDS, made in DEPC treated water) is added, followed by 1 volume of phenol (equilibrated in 10 mM Tris-HCl pH 8.0) heated to 65°C. The samples are mixed by vortexing and incubated at 65°C for 15 min, vortexing every 5 min. After phase separation by centrifugation at room temperature for 5 min, the aqueous phase is re-extracted with phenol, re separated by centrifugation and re-extracted with chloroform/isoamyl alcohol. To the aqueous phase are then added 0.1 volume of DEPC-treated sodium acetate (pH 5.0) and 2 volumes of ethanol. The RNA is recovered by precipitation at -70°C, followed by centrifugation at 4°C for 15 min. The samples were then analysed by agarose gel electrophoresis as described in example 1.

After blotting to Zeta-Probe membrane (BioRad), the hybridization protocols were as above for Example 2.

C. Total protein from HaSV - electroporated protoplasts.

Protoplasts were analysed by SDS-polyacrylamide gel electrophoresis and Western blotting as described in Example 1.

Results

i) Use of complete (replication-competent) RNA virus genome in protoplasts

a) HaSV replication in protoplasts

The nodavirus FHV has previously been shown to replicate in barley protoplasts (Selling H.H., Allison, R. F. and Kaesberg, P. Proc. Natl. Acad. Sci. USA 87,434-8 (1990). To determine whether HaSV virus RNA can replicate in plants protoplasts, when introduced by electroporation, experiments using protoplasts from *Nicotiana plumbaginifoli* and wheat have been conducted. (These are all species for which protoplasts are regularly available in the Division of Plant industry). Assays for replication including RNA (Northern) blots using probes derived from cloned fragments of cDNA to RNAs 1 and 2 (SEQ ID Nos: 39 and 47), and Western blots, using the antiserum to purified HaSV particles. Initial experiments showed that both HaSV virus and RNA electroporated into protoplasts of *N. plumbaginifolia* resulted in HaSV replication as studied using and verified by northern blots and ELISA. As a positive control TMV RNA was electroporated and was replication observed.

b) Bioassays

Protoplasts into which HaSV RNA had been introduced by electroporation were harvested after 6 or 7 days post electroporation and used in bioassays on neonate larvae by addition to normal diet. The results showed significant stunting of test larvae in comparison to control larvae (see Table 1 below). Protoplasts lacking HaSV RNAs had no effect on the larvae, confirming the result of control experiments. This result confirms that HaSV RNA, when expressed or replicated in plant cells, is able to cause the formation of infectious virus particles able to control insect larvae feeding on the plant material.

Northern blotting has been used to confirm that RNA electroporation into protoplasts leads to RNA replication.

Table 1: Results of Bioassay from a typical experiment with *Nicotiana* and oat protoplasts (oat results are shown in brackets) [see over]

	Treatment	Number	Escapes	Number stunted
1.	diet only	12 (12)	2 (3)	0/10 (0/9)
2.	diet+protoplasts	12 (12)	0 (1)	0/12 (0/11)
3.	HaSV+diet	12 (12)	0 (1)	12/12 (11/11)
4.	diet+HaSV/protoplasts	12 (n.d.)	0 (n.d.)	12/12 (n.d.)
5.	diet+RNA/protoplasts	12 (12)	0 (0)	11/12 (10*/12)

* HaSv replication in the larvae was confirmed except for two larvae which were dead. The letters "n.d." mean the experiment was not done.

The above results demonstrate assembly of HaSV particles from electroporated RNA in protoplasts of both moncot and dicot plant species.

c) Plasmids to test replication of cloned and engineered forms of HaSV

(1) Plasmids allowing in vitro transcription of HaSV RNAs 1 and 2 (SEQ ID Nos: 39 and 47) for electroporation into protoplasts have already been described above.

(2) Plasmids for transient expression of individual HaSV RNAs (1 or 2) (SEQ ID Nos: 39 and 47) in protoplasts. Full-length cDNAs for the two viral RNAs have been inserted into expression plasmids pDH51 (with the CaMV 35 S promoter. Pietrzak M., et al (9186) Nucl. Acids Res. 14, 5857-68) for dicots and pActI.cas (with the rice actin promoter) for monocots (McElroy et al (1990) The Plant Cell 2: 163-171). As with the vectors for expression in insect cells, these expression plasmids are being modified to include a cis-acting ribozyme for generation of authentic ends. The non-ribozyme plasmids gave no virus replication.

ii) Expression of capsid protein in plants

In view of the present inventors' observation that empty particles ("assembled capsids") are being produced in baculovirus-infected cells that efficiently express the P71 precursor gene, expression of the coding region for the capsid protein in tobacco plants was investigated. The vector chosen for this purpose is based on pDH51 which carries the CaMV 35S promoter and polyadenylation signal. If necessary for improved expression, this vector can be modified by the addition of a translation enhancer sequence from e.g. TMV. Although certain groups have constructed transgenic plants expressing the capsid proteins of plant viruses, there has been only one recent report of assembly of empty capsids in such plants (Bertioli et al.,(1991) J. gen. Virol. 72: 1801-9). Bertioli et al point out that the protein-protein interactions in most icosohedral plant RNA viruses may be too weak to allow assembly of such capsids. In addition to the present inventors' observation of empty HaSV capsids, it has been found these capsids are very tough, showing great resilience to e.g. repeated cycles of freezing and thawing, so that it is expected to see assembly of empty HaSV capsids ("assembled capsids") in transgenic plants.

Construction of capsid protein expression plasmid.

Vector used was pDH51; linearised with BamHI and phosphatased.

Insert was PCR product made using following 2 primers:

CAPPLANT:

5' GGGGATCC ACA ATG GGA GAT GCT GGA GTC -3'

(BamHI)

(i.e. A BamHI site followed by plant consensus context for ATG of capsid protein gene and 15 further nucleotides of this gene - nts 366-383 of HaSV RNA2).

HVP6C2 (Example 3)

The PCR product was made with VENT polymer (New England Biolabs). After gel purification, it was cut with BamHI and cloned into the vector. Orientation screened with EcoRI to identify insert in same direction as promoter giving plasmid pDHVCAPB. Expression was verified by Western blotting using anti-HaSV antiserum. Both precursor P71 and processed P64 capsid protein were detected in protoplasts following transfection with pDHVCAPB, showing assembly of virus-like particles.

EXAMPLE 6

IDENTIFICATION OF MIDGUT BINDING DOMAINS

Materials & Methods

A. Plasmid construction

Was as described in Examples 3 and 4.

B. Western blotting

Was as described in Examples 1 and 3.

C. Invitro translation

In vitro transcripts of cloned CDNA of HaSV RNA's was translated in vitro as in Examples 1 and 3.

D. Preparation of Brush Border Membrane Vesicles.

Brush Border Membrane Vesicles were prepared from freshly isolated larvae midguts of *H.Armigera* by the method of M.Wolfersberger et al (1987) Comp. Biochem. Physiol. 86A: 301-308, as modified by S.F.Garczyuski et.al. (1991) Applied Environ. Micro-biol 57: 1816-2820. Brush Border Membrane Vesicles binding assays using invitro labelled protein or ^{125} I-labelled protein were as described in Garczynski et.al. (1991) or in H.M.Horton and Burand, J.P. (1993) J.Virol. 67: 1860-1868.

Results

i) Determination of epitopes on the capsid surface

Comparison of the recently published sequence of the *Nudaurelia* ω virus (*N*wV) capsid protein with that of HaSV shown that these proteins are closely related and fall into four distinct domains, which are alternatively variable and highly conserved. These domains are summarised as follows:

Residues:	HaSV	1-49	50-272	273-435	437-647
	*N*ωV	1-46	47-269	270-430	431-645
% identity:		37	81	34	81

Comparison of this observation with the alignment by Agrawal and Johnson (1992) between the *N*wV and the nodavirus BBV (whose crystal structure is known: Hosur et al (1987) Proteins: Structure, Function & Genetics 2: 167-176) showed that the variable region coincided with a region forming the most prominent surface protrusion on the BBV capsid. Both HaSV and *N*wV carry large insertions at this point relative to BBV, and these insertions are largely different in sequence. Assuming that the alignment by Agrawal and Johnson (1992) is correct, then this means that HaSV and *N*wV have a more prominent pyramid-like structures as a surface protrusion than do the nodaviruses, and the pyramid-like structures are different. As already noted, there is no immunological cross-reactivity between the two viruses, despite the high degree of identity. There is thus a strong implication of the variable domain as a surface protrusion which functions as the sole antigenic region.

To confirm this a 400 bp NarI fragment spanning the variable region was deleted from the capsid gene in the expression vector. With end-filling of these sites the deletion is in-frame, so that a truncated protein of ca. 57 KDa is produced in bacteria upon induction. This protein was recognized only poorly on Western blots by the antiserum against intact HaSV particles made in rabbits. The central variable domain was recognized well by the antiserum when expressed in isolation from the rest of the capsid gene.

As shown in the table above the region of HaSV capsid protein comprising residues 273-439 shows great divergence form the corresponding region of the NwV capsid protein, compared to its immediate flanking regions. Within this region an especially divergent domain is found from residue 351 to residue 411, which shows only 25% identity to the corresponding region of the NwV capsid protein. This region is flanked by the sequences corresponding to the b-sheet structural features b-E(residues 339-349) and b-F(residues 424-431) of the HaSV capsid protein, based on the alignment the NwV and nodavirus capsid proteins by Agrawal and Johnson (1992), and is therefore likely to form the loop of the most prominent surface protrusion on the HaSV capsid. This is based on comparison and correspondence to the nodavirus capsid protein structure and capsid structure as described by Wery J.-P. and Johnson, J.E. (1989) Analytical Chemistry 61, 1341A-1350A and Kaesberg, P., et al. (1990) J. Mol. Biol. 214, 423-435. This loop is thought to contain important epitopes. It is significant that this exterior loop on the nodavirus capsid protein is one of the most variable regions when capsid proteins sequences from a number of nodaviruses are compared (Kaesberg et al. 1990).

Finally, the present inventors have observed a significant level of immunological cross-reaction on Western blots, between antisera against the CryIA(c) Bt toxin and HaSV capsid protein, whether obtained from virus or expressed in bacteria. Initial data from the NarI deletion mutant described above suggest that this binding is not to the central variable domain, but to other regions of the capsid protein. The only other region of the proteins which shows extensive sequence variability, the amino terminus, cannot be responsible for the binding, since both authentic capsid protein and the protein with an altered amino terminus expressed in bacteria are recognized by the anti Bt antisera.

ii) In-Vitro binding assays

The full-length clones for *in vitro* translation yielding highly ^{35}S or ^{3}H labelled proteins were constructed by replacing the bacterial translation interaction signal in the T7

plasmids above by the more active eucaryotic context sequence from the JHE gene. The labelled capsid protein made by *in vitro* translation of the *in vitro* transcripts may be tested for binding to brush border membrane vesicles (BBMV's). Conditions are optimised by testing different procedures. The deletion mutant lacking approximately 125 amino acids in the central region, and containing the variable domain, as well as others derived from it are also tested.

iii) Fusion proteins comprising virus capsid midgut binding domains and other proteins

The idea behind these tests is to fuse the binding domain from the HaSV capsid protein to either large proteins (preferably indigestible, causing protein to aggregate in or on the midgut cells) or toxin domains from other proteins with suitable properties but normally different binding specificities (e.g. Bt). In initial experiments, the gene for the complete capsid protein has been fused to the GUS gene, as has a deletion mutant containing essentially only the central portion of the capsid gene. The resulting fusion proteins are being expressed in bacteria and tested for GUS activity, and makes them sensitive probes for binding experiments on midgut tissue.

iv) Mapping binding sites using Bt/HaSV fusion proteins

Analysis of deletion mutants of the CryIA(c) Bt toxin has identified domains which may be involved in determining the host-specificity of this Bt by acting as receptor-binding sites (Schnepf et al (1990) J. Biol. Chem. 265: 20923-20930; Li et al (1991), Nature 353: 815-21. The present inventors have obtained a clone of this toxin gene. Deletion mutants corresponding to those identified by Schnepf et al are constructed. Segments of the HaSV capsid protein gene can then be inserted into these mutants, the protein expressed in bacteria and their insecticidal function assayed.

EXAMPLE 7

VIRAL GROWTH IN CELL CULTURE

Materials & Methods

A. Cell Lines

The following cultured insect cell lines were tested for infection by HaSV: *Drosophila melanogaster, Helicoverpa armigera* (ovarian derived), *Heliothis zea* (ovarian derived), *Plutella xylostella, Spodoptera frigiperda (SF9).*

All lines were grown under standard conditions. Upon reaching confluence, the culture medium was removed and all mono-layers covered with 1.5 ml of cell culture medium into which HaSV had been diluted; the average multiplicity of infection (M.O.I.) was 10^4. After adsorption at 26°C for 2h, the inoculum was removed, the cells carefully washed twice with phosphate buffered saline (pH 7.0) and incubation continued with 5 ml of 10%. Foetal calf serum in TC199 culture medium (Cyto Systems).

B. Northern Blotting Analysis.

Virus replication in all the above cell lines was confirmed by northern blotting analysis. Total RNA was extracted from infected cells by the method of Chomczynski and Sacchi (1987). Anal. Biochem. 162: 156-159. The cells were lysed in 1 ml of lysis solution (4M guanidinium thiocyanate, 25mM sodium citrate, pH 7, 0.5% sarcosyl, 0.1M 2-mercaptoethanol). In order, 0.1 ml of 2M sodium acetate, pH 4, 1 ml of phenol (0.2M sodium acetate equilibrated), and 0.2 ml of chloroform-isoamyl alcohol mixture (49:1) were added with thorough mixing between reagents. This was then vortexed for 10 s and cooled on ice for 15 min. Tubes were centrifuged in an Eppendorf centrifuge at 14k for 15 min at 4°C for at least 15 min to allow RNA precipitation. RNA was pelleted by centrifugation at 14k for 15 min, washed with 0.6 ml of ice-cold 70% ethanol, pelleted once again (10K, 10 min), air dried at room temperature and resuspended in DEPC (Sigma) treated millipore water. RNA was subject to denaturing agarose gel electrophoresis in the presence of formaldehyde according to Sambrook et.al. (1989). The gel was Northern transferred to a zeta-probe membrane (Biorad) as described by Sambrook et.al. (1989). The probe was prepared by random-priming the 3' sequences of the HaSV genome using DNA and cDNA clones pSHVR15GB and pT7T2p71SR-1 as per manufacturer's instructions (Boehringer-Mannheim). Hybridization was carried out as described for the standard

DNA probe protocol contained within the literature for the zeta-probe membrane (Biorad).

C. Vectors

Vectors as described below.

Results

It has been found that HaSV will replicate in several continuous cell lines, of which the best is the *Spodoptera frugiperda* line SF9. Time course assays by Northern blotting in SF9 cells have shown that RNA 1 (SEQ ID No: 39) replication is clearly detectable within a few hours of infection. RNA 2 (SEQ ID No: 47) is present only in very small amounts early in infection and accumulates much more slowly than RNA 1 (SEQ ID No: 39) does. This observation is consistent with one made earlier in HaSV-infected larvae, where RNA 2 (SEQ ID No: 47) replication was not observed until 3 days after infection.

Some apparent replication was also observed in Drosophila cells (DL2), but with the difference that more RNA 2 (SEQ ID No: 47) replication was observed at the early time points compared to the lepidopteran cell lines above.

Plasmids that express the HaSV genome as RNA transcripts from full length cDNA clones have been constructed and tested. These clones, constructed by PCR and carefully checked, have restriction sited immediately adjacent to the ends of the sequence. Transcription is driven from a specially-re-engineered Drosophila HSP70 promoter.

i) Constructs for expression in insect cells

The constructs are based on vectors carrying the Drosophila HSP 70 or actin promoters and suitable polyadenylation signals from Drosphila (Corces & Pellicer (1984) J. Biol. Chem. 259: 14812-14817) or SV40 (Angelichio et al (1991) Nucl. Acids. Res. 18: 5037-5043). Since transcription from such plasmids generates viral RNAs carrying long 3' terminal extensions derived from sequences in the poladenylation signal fragment, it is necessary to achieve cleavage of the transcript immediately after the 3'sequence of the viral RNA. These plasmids gave no virus replication, presumably because of the 3' terminal extension. The method of choice for obtaining authentic 3' termini is based on introduction of DNA sequences encoding a cis-acting ribozyme into the constructs. With suitable engineering, such a ribozyme will cleave immediately 3' to the viral sequences within the transcript. Suitable ribozymes, based on the hepatitis delta virus (Been M.D., Perrotta, A. T. & Rosenstein, S.P. Biochemistry 31, 11843-52 (1992) or the hairpin cassette ribozyme (Altschuler, M., Tritz R. & Hampel, A. Gene 122, 85-90 (1992) have been designed (see Example 4). This involves synthesis of overlapping oligonucleotides, which are then annealed and end-filled with the Klenow fragment of DNA polymerase, to create short DNA fragments encoding the desired ribozyme. These fragments carry restriction sites at their termini allowing them to be ligated into plasmids between the viral RNA cDNA (which has a 3' restriction site added by PCR) and the restriction fragment carrying the poladenylation signal. Ribozyme function has been verified (Example 9).

The Drosophila HSP70 promoter was joined to the HaSV RNA 1 sequence as follows. A BamHI restriction site was introduced into the promoter sequence as described on p.5 of this specification. Oligonucleotide HVR1B5P described in Example 8 was used to prime PCR of RNA 1 to yield a cDNA copy of the RNA carrying a BamHI restriction site 5' to the RNA 1 sequence and separated from it by the nucleotides ACA which end the HSP70 promoter just before the start of transcription. This common BamHI site was used to link the HSP70 promoter and the HaSV RNA 1 sequence. The resulting plasmid was completed by adding either the hairpin cassette ribozyme

(giving plasmid pHSPR1HC) or the HDV ribozyme (giving plasmid pHSPR1HDV) plus the SV40 late polyadenylation sequence.

A similar approach was used to obtain plasmids for RNA 2 i.e. pHSPR2HC and pHSPR2HDV.

An alternative approach is to link the promoter and the HaSV cDNAs using blunt end ligation of a DNA fragment and carrying the promoter and terminating at the last nucleotide before the start of transcription (the underlined residue in AC<u>A</u>) and the cDNA fragments corresponding to either HASV RNA 1 or 2, as described for the plant expression plasmids in Example 9.

The latter approach was used to join the sarcoma virus (RSV) long terminal repeat (LTR) promoter to the HaSV cDNAs for expression in insect cells. The RSV LTR promoter is active in many animal cells (Cullen, B.R. Raymond, K. & Ju, G. (1985) Mol. Cell. Biol. 5,438-447) and also in lepidopteran cell lines (D. Miller personal communication). It was obtained from plasmid pRSVCAT (Gorman, C., Padmanabhan, R. & Howard, B.H., (1983) Science 221, 551-553) as a 495 bp fragment carrying a 5'-XbaI site (added by PCR) and terminating at a blunt end with the sequence AA<u>C</u>, with the underlined residue corresponding to that immediately before the start of transcription. The resulting plasmids, pRSVR1HCLA and pRSVR2HCLA, carry the HaSV RNA 1 and 2 cDNAs, respectively, and are otherwise like pHSPR1HC and pHSPR2HC, respectively. These plasmids carry the SV40 late polyadenylation signal. They allow efficient and precise expression of the HaSV genomic RNAs in insect cells, for example if introduced using a baculovirus vector or by transfection.

EXAMPLE 8

SHEDDING OF INFECTED CELLS

Materials & Methods

A. Confocal Laser Scanning Microscopy. (CLSM)

CLSM enables the visualisation and analysis of three-dimensional cell and tissue structures at the macro and molecular levels. The Leica CLSM used in this example is based on an MC 68020/68881 VME bus (20MHz) with standard 2Mbyte framestore and 4Mbyte RAM and OS9 operating system with programmes written in C code. It incorporates a Leica Diaplan research microscope and using X10/0.45, X25/0.75,X40/1.30 and X63/1.30 Fluotar objectives has a claimed optical efficiency better than 90%. The confocal pinhole is software controlled over the range of 20 to 200 mm. Excitation at 488 and 514 nm is provided by a 2 to 50 mW argon-ion laser.

B. Immunocytochemistry (ICC).

For whole mount ICC, tissues were dissected under saline and fixed in fresh 4% formaldehyde in phosphate buffered saline (PBS) for at least 15 mins. After multiple washes in PBS they were permeablized either by 60 mins incubation in PBT (PLBS with 0.1% Triton X-100 plus 0.2% bovine serum albumin). After 30 mins blocking in PBT+N (5% normal goat serum) tissue was incubated in primary antibody diluted (1:40) in PBT+N for at least 2 hrs at room temperature then at 4°C overnight. After extensive washing in PBT and 30 mins blocking in PBT+N the FITC conjugated secondary antibody diluted (1:60) in PBT+N was incubated for 2 hrs at room temperature plus overnight at 4°C. After multiple washes in PBT and PBS the tissue was cleared in 70% glycerol and mounted in 0.01%w/v p-phenylenediamine (Sigma#P1519) dissolved in 70% glycerol. All processing was at room temperature unless otherwise stated.

Results

The inventors' current model for the effect of HaSV involves the detection by the insect midgut of infected cells, their identification as infected and their subsequent shedding in numbers sufficient to cause irreparable damage to the insect midgut. The evidence for this is based on the above and on the following direct observation of the fate of infected cells in midgut tissue over 1-3 days post infection. These results in repeat experiments were complicated by the discovery that another unrelated virus was present in the larval population being tested. Preliminary findings indicated that HaSV infection activates or facilitates pathogenesis of the unrelated virus and together these cause severe disruption of the larval gut cells. Thus these two agents appear to act synergistically in causing gut cell disruption.

Midguts from larvae infected with HaSV were treated with the antiserum to purified HaSV particles (above) and examined under the Laser confocal microscope (described above). This established that some midgut cells were sufficiently infected with HaSV to give strong fluorescence signals. Such cells were moreover clearly separating from the surrounding tissue, a sign that they were in the process of being shed.

Similar observation have been made with other insect viruses (Flipsen et al (1992) Society for Invertebrate Pathology Abstract #96) although in these cases the effect is too localised and weak to cause any anti-feeding effect apparently only the small RNA virus of the tetraviridae which are localised to the gut and cause more-or-less severe anti-feeding effects in their hosts (Moore, N.F. in Kurstak E. (Ed) (1991) Viruses of Invertebrates. Marcel Dekker, New York pp277-285) are capable of such an effect to an extent sufficient for pest control.

Following on from the immune-fluorescence work, *in situ* hybridization can be carried out to detect RNA replication in infected cells.Furthermore, larvae infected with a recombinant HaSV expressing a foreign gene at early stages (by insertion of that gene into RNA 1 in place of the N-terminal portion of the replicase gene) can be studied. A correlation between virus replication and cell rejection can be confirmed by

histochemical analysis of the midgut cells of the infected larvae. Thus the cell-shedding phenomenon offers a direct and rapid assay for early events in HaSV-infected gut tissue. Extracts of baculo-vector infected insect cells carrying empty HaSV particles can be fed to larvae directly and the midgut examined by toluidine blue staining and immune-fluorescence at intervals after infection. This will allow direct determination of whether the particles can bind the brush border membranes in intact gut, and whether such binding can induce the massive disruption evident in normally infected larvae. Control experiments using extracts from cells infected with the baculovector alone can be conducted to observe and distinguish effects due to the vector. The immune-fluorescence assay on midgut tissue allows analysis of binding to midgut brushborder membranes. Once determined for wild-type capsid protein expressed from a baculo-vector, deletion or replacement mutants can be inserted into the baculovectors. Suitable cell extracts from these can be used to infect larvae.

EXAMPLE 9

ENGINEERED VIRUS AND USES

Materials & Methods

(as indicated in earlier Examples)

i) Engineered virus as a vector for other toxin genes

This involves placing suitable genes under control of HaSV replication and encapsidation signals. Genes which may be suitable include intracellular insect toxins such as ricin, neurotoxins, gelonin and diphtheria toxins. The toxin gene may be placed in the viral gene such that it is a silent (downstream) cistron on a polycistronic RNA, or in a minus strand orientation, requiring replication by the viral polymerase to be expressed. Standard techniques in molecular biology can be used to engineer these vectors.

A discussion of two recombinant HaSV vectors which have been designed is given below:

for RNA 1 (SEQ ID No: 39):

The reporter gene (or one of the toxin genes mentioned above) is inserted in place of the amino-terminal portion of the putative replicase gene, such that the intiation codon used for the replicase (ie that at nucleotides 37-39 of the sequence) is now used to commence reporter gene translation. The fusion is achieved by the use of artificial NcoI restriction sites common to both sequences.

The short 36 nucleotide 5'-untranslated leader of RNA 1 (SEQ ID No: 39) (shown in upper case) is synthesised as the following sequence:

ggggatccacaGTTCTGCCTCCCCCGGACGGTAAATATAGGGGAACCATG Gtctagagg, (SEQ ID No: 53)

using two overlapping oligonucleotides comprising the first 31 (oligonucleotide HVR1B5P) nucleotides and the complement of the last 40 nucleotides (oligonucleotide HVR1NCO) respectively. These primers are annealed and end-filled by Klenow. The resulting fragment is then cut with BamHI and Xbal (sites underlined) and cloned with plasmid vector pBSIISK(-) to give pBSSKR1NCO.

The GUS gene carrying a Ncol site at the ATG codon was obtained as a NcoI-SacI fragment from plasmid pRAJ275 (Jefferson, RAJ Plant Mol. Biol. Rep 5, 3387-405 (1987)). This SacI site is located just downstream from the coding sequence for the GUS gene.

The 5' leader of HaSV RNA1 is excised as a BamHI-NcoI fragment from the plasmid pBSSKR1NCO, and is ligated together with the NcoI-SacI fragment carrying the GUS gene into plasmid pHSPR1HC or pHSPR1HDV or pDHStuR1HC carrying the full-length cDNA insert of RNA 1 (see above) which has been cut with BamHI and SacI. The resulting plasmid then carries a complete form of RNA 1 (SEQ ID No: 39) but with the amino-terminal portion of the replicase gene substituted by the GUS gene. It is desirable to produce a construct with approximately the same size as RNA 1 (SEQ ID No: 39) for encapsidation purposes.

Similar approaches are adopted for RNA 2 (SEQ ID No: 47), with the foreign, reporter or toxin gene fused to the initiation codon of either P17 or P71. In either case the context sequence of the introduced gene is modified to give the necessary expression

level of that protein. The foreign gene is introduced into plasmids pHSPR2HC or pHSPR2HDV or pDHStuR2HC.

The above recombinants have been described specifically as insertions of a reporter gene (GUS). The toxin genes to be inserted are described on page 14 of the specification. These preferably further require a signal peptide sequence added at the amino-terminus of the protein.

ii) Capsid technology

Identification of encapsidation (and replication) signals on virus RNA allows design of RNAs which can be encapsidated in HaSV particles during assembly of virus in a suitable production system. The virus capsids then carry the RNA of choice into the insects midgut cells where the RNA can perform its intended function. Examples of RNAs which may be encapsidated in this manner include RNAs for specific toxins such as intracellular toxins, such as ricin, gelonin, diptheria toxins or neurotoxins. This strategy is based on the resistance of the virus particle to the harsh gut environment.

iii) Other uses of the capsid particle

The capsid particles can be used as vectors for protein toxins. Knowledge of icosahedral particle structure elucidated by the inventors suggests that the amino and especially the C-termini are present within the capsid interior. It is possible to replace or modify the amino acid sequence corresponding to P7 such that it encodes a suitable protein toxin which is cleaved off the bulk of the capsid protein during capsid maturation. As with toxin-encoding mRNAs, the HaSV capsid delivers it to the midgut cell of the feeding insect, where it exerts the desired toxic effect.

iv) Use of HaSV in plants

The use of HaSV in the production of insect-resistant transgenic plants are shown in Fig. 12. These inventions are based on the use of either the complete HaSV

genome, or of the replicase gene as a tool for the amplification of suitable amplifiable mRNAs (e.g. encoding toxin) or of the capsid protein as a means to deliver insecticidal agents. These strategies are now described in some detail.

a) Use of the complete HaSV genome

Fragments of cDNA corresponding to the full-length HaSV genome components RNAs 1 and 2 (SEQ ID Nos: 39 and 47) are placed in a suitable vector for plant transformation under the control of either a constitutive plant promoter (e.g. the CaMV 35S promoter mentioned above) or an inducible promoter or a tissue specific (e.g. leaf-specific) promoter. The cDNAs are followed by a cis-cleaving ribozyme and a suitable plant polyadenylation signal. Transcription and translation of these genes in transgenic plant tissues and cells leads to assembly of fully infectious virus particles to infect and kill feeding larvae.

The following experiments were conducted. The plasmids for expression used the CaMV 35S promoter to generate transcripts commencing at the first nucleotide of the HaSV RNAs 1 and 2 (SEQ ID Nos: 39 and 47). The vector pDH51 (M. Pietrzak, R. Shilito, T. Hohn and I. Potrykus (1986). Nucleic Acids Research 14, 5857) which carries the CaMV 35S promoter followed by a multiple cloning site and the CaMV polyadenylation fragment was modified to make a suitable vector, pDH51Stu, carrying a StuI site at the immediate 3' end of the CaMV 35S promoter. The promoter thereby terminates in the sequence GAGAGGCCT, with the underlined residue being that at which transcription would start. (Similar vectors have been described by Mori *et al.*, J. General Virology **72**, 243-246 (1991) and Dessens and Lomonossoff, ibid 74, 889-892 (1993).) The StuI site (AGG/CCT) is followed by a BamHI site (GGATCC). Cleavage of this vector with StuI and BamHI generates a vector DNA molecule with one blunt end (from StuI cleavage) and one sticky BamHI end. This allows ligation of cDNA molecules corresponding to the full-length HaSV genomic RNAs, and carrying a blunt end at the 5' end of the full-length cDNA and a BamHI site after the 3'-end of the full-length cDNA.

Suitable cDNA fragments carrying a blunt end corresponding to the 5'-terminal nucleotide of either RNA 1 or 2 (SEQ ID Nos: 39 and 47) were generated using PCR and an oligonucleotide primer corresponding to the 5'-terminal first 18 nucleotides of the sequence of either RNA 1 (SEQ ID No: 39) or RNA 2 (SEQ ID No: 47). The cDNA sequence corresponding to the 3' terminal sequences of either RNA 1 (SEQ ID No. 39) or RNA 2 (SEQ ID No 47) were followed on these DNA fragments by sequences corresponding to one of the ribozymes whose sequences are shown in Fig. 8 and whose construction is described in Example 7. The 3'-terminal sequence corresponding to an XbaI site (TCTAGA) shown in these ribozyme sequences was followed on the suitable DNA fragments by a BamHI site, which upon cleavage with this enzyme yielded a sticky end capable of being ligated into the BamHI end of the vector cleaved as described above. There were therefore a total of four suitable DNA fragments for insertion into the vector:

RNA 1 (SEQ ID No: 39) followed by the hairpin cassette (HC) ribozyme
RNA 1 (SEQ ID No: 39) followed by the hepatitis delta virus (HDV) ribozyme
RNA 2 (SEQ ID No: 47) followed by the hairpin (HC) ribozyme
RNA 2 (SEQ ID No: 47) followed by the hepatitis delta virus (HDV) ribozyme.

These four fragments were individually ligated into the vector pDH51Stu cleaved with StuI and BamHI to generate four distinct plasmids as follows:

pDHStuR1HC
pDHStuR1HDV
pDHStuR2HC
pDHStuR2HDV

Transcription from the 35S promoter in these plasmids results in RNAs commencing at the first nucleotide of either the RNA 1 sequence (SEQ ID No: 39) or RNA 2 sequence (SEQ ID No: 47) and terminating in the CaMV polyadenylation fragment. Self-cleavage at the locations shown in Fig. 8 by the cis-acting ribozymes obtained within these transcripts generates RNA molecules with the 3'-termini corresponding to the natural virus termini.

After amplification and purification on CsCl gradients, thirty mg of each of these four plasmids was transfected by electroporation into aliquots of two million *N. plumbaginifolia* protoplasts (as described in Example 5) either individually or in the combinations listed below:

pDHStuR1HC + pDHStuR2HC

pDHStuR1HDV + pDHStuR2HDV

The production of infectious HaSV particles within transfected protoplasts was then demonstrated by bioassay on heliothis larvae. After incubation at 25°C for 3-5 days, the protoplasts were recovered by low speed centrifugation and applied directly to standard heliothis diet as surface contamination for bioassay as described in Example 1. Stunting was only observed when plasmids expressing HaSV RNA 1 (SEQ ID No: 39) and RNA 2 (SEQ ID No: 47) were co-transfected, and then only in the case of those carrying the hairpin ribozyme to generate the viral 3' ends (see Table 2). In contrast, constructs carrying the HDV ribozyme at the 3' end were not infectious. The reasons for this have not been determined. As expected, expression of RNA 1 or 2 (SEQ ID Nos: 39 and 47) alone in protoplasts did not lead to the assembly of infectious particles. Western blot analysis of protoplasts transfected with the RNA 2 (SEQ ID No: 47) constructs did show production of limited amounts of the capsid protein.

Suitable control experiments confirmed that larval stunting was due to HaSV particles generated *de novo* in the protoplasts. As shown in the Table 2, neither the protoplasts alone nor protoplasts mixed with plasmid DNA were capable of initiating stunting.

Table 2

Treatment		No. of larvae	Escapes	No. stunted
1.	diet alone	24	0	0
2.	diet + HaSV	24	0	24
3.	diet + protoplasts	24	0	0

4.	diet + pDHStuR1HC	24	0	0
5.	diet + pDHStuR1HDV	24	0	0
6.	diet + pDHStuR2HC	24	0	0
7.	diet + pDHStuR2HDV	24	0	0
8.	diet + pDHStuR1HC + pDHStuR2HC*	24	0	22
9.	diet + pDHStuR1HDV + pDHStuR2HDV*	24	0	0
10.	pDHStuR1HC + pDHStuR2HC (but mixed with protoplasts)	24	0	0

* these plasmids were co-transfected with pDHVCAPB (see Example 5)

HaSV infection of stunted larvae was confirmed by dot-blotting of RNA using HaSV specific probes. After weighing, larva were sacrificed and total RNA extracted as follows. Each larva was homogenised in the presence of 260 ml deionised water, 24 ml 2M sodium acetate pH 4.0 and 200 ml phenol equilibrated with 2M sodium acetate pH 4.0. After centrifugation at 14 000 rpm for 15 min at 4°C, the supernatant (about 200 ml) was removed and extracted once with an equal volume of chloroform. After centrifugation, the supernatant (about 200 ml) was mixed with 20 ml of sodium acetate and 400 ml of absolute ethanol. The precipitate after centrifugation was vacuum dried and redissolved in 5-10 ml of sterile, DEPC-treated water. For dot-blotting, the RNA was mixed with 70 ml of DEPC-treated water and 30 ml of 10 mM EDTA, 30 mM NaOH. HaSV RNA was determined and quantified by dot blotting (as described in Example 2) using a probe random primed DNA from clones corresponding to the terminal 1000 nucleotides of RNA 1 and 2. All larvae recorded as stunted in the bioassays were found to carry HaSV and give signals comparable to those of the larvae fed purified HaSV particles (Table 2). To confirm that the larvae were infected with HaSV, ten aliquots of protoplasts were electroporated with plasmids pDHStuR1HC + pDHStuR2HC and the protoplasts fed (after incubation) to 150 heliothis larvae. The larvae were allowed to grow for one week, upon which significant stunting was observed in 50% of the larvae, and virus was then purified from these stunted larvae as described in Example 1. Analysis on CsCl gradients showed the production of distinct

the RNA can be encapsidated by HaSV capsid protein and/or replicated by the HaSV replicase in infected insect cells (see Figs. 12a and 12b)

Transgenic plants would contain two different transgenes, making either unmodified capsid protein precursor or a modified form in which most of the carboxyterminal protein P7 is replaced by a suitable insect-specific toxin or one which is inactive as part of a fusion protein. (Gelonin or other ribosome-inactivating proteins, insect gut toxins or neurotoxins may be suitable here.) Expression from these two transgenes would be regulated so that only the required amounts of the modified and unmodified forms are made in the plant cell, and assembled in such proportions into the capsoids. One way to modulate the production of capsotixin fusion proteins is to make translation of the carboxyterminal toxin reading frame dependent on a translational frameshift or read-through of a termination codon. With an appropriate low frequency of frame-shifting (eg 0.1 - 2%), it could even be sufficient to use a single transgene, if it were possible to synthesise the P7 portion and the toxin portion as overlapping genes. Upon assembly (which we have demonstrated in insect cells using the baculovirus vectors) and maturation, the protein precursors are cleaved and release the mature P7 and the toxin, which remain within the capsoids. These proteins are not released until capsoid disassembly occurs in insect gut cells. The processed form of the toxin is then able to kill the pest.

(c) HaSV particles devoid of nucleic acid carrying one or more suitable protein toxins and/or their mRNA

A protein toxin (or toxins) is expressed as a fusion with the capsid protein. The fusion protein then assembles into capsid carrying the toxin(s). These capsids present in the plant tissue exert an antifeeding effect on insects attaching the plant.

EXAMPLE 10

EXPRESSION OF HaSV IN OTHER DELIVERY VECTORS

Materials & Methods

(as indicated in earlier Examples)

Constructs similar to those for plant expression are introduced into yeast or bacteria by standard techniques. Virus particles are assembled for either fully infectious virus or any of the modified or biologically contained forms described in Example 9. Microbes produced in suitable fermentation or culture facilities and carrying such forms of the virus are then delivered to the crop by spraying. The microbial cell wall provides extra protection for the virus particles produced within the microbe.

Well established techniques exist for culture and transformation of yeast (Ausubel, F.M. *et al.* (eds) Current Protocols in Molecular Biology. J. Wiley & Sons, NY, 1989). An example of a yeast expression vector is pBM272, which contains the URA3 selectable marker (Johnston, M. & Davies, R.W. Mol. Cell. BIol. 4, 1440-8, (1984); Stone, D. & Craig, E. Mol. Cell. Biol. 10, 1622-32 (1990). Another example of an expression vector is pRJ28, carrying the Trp1 and Leu2 selectable markers.

Yeast has recently been shown to support replication of RNA replicons derived from a plant RNA virus, brome mosaic virus (Janda, M. & Ahlquist, P. Cell 72, 961-70 (1993). Since the BMV replicase is distantly related to that of HaSV, and the two viruses are likely to replicate by similar strategies within cells, yeast cells probably contain all the cellular factors required for HaSV to generate infectious virus.

For bacteria, suitable expression vectors have been described above.

EXAMPLE 11

The transvirus approach for insect pest control: Making transgenic plants expressing HaSV

1. Vector construction

A special binary vector was constructed for transforming plants with the HaSV genome. This vector is based on pART27 (A. Gleave (1992) Plant Mol.Biol.**20,** 1203-1207), which was modified to (1) carry an alternative origin of replication for the host

Agrobacterium tumefaciens and (2) incorporate restriction sites in the multiple cloning site for restriction enzymes Asc I and Pac I which recognise rare (8bp) sequences.

For engineering the multiple cloning site, pART27 was cut with SpeI and NotI. Ten picomoles of each of the two oligos whose sequence follows (TOP and BOTTOM) were annealed in 10 microlitres of water (heated to 80°C for 2 min and allowed to cool slowly to room temperature). The sticky ends on these annealed oligonucleotides allowed the insert to be cloned into pART27 (giving pART27mod) as described in Example No. 3 and 9.

Sequence of oligonucleotide:

TOP: 5'-GGCCGCTTAATTAAGGATCCGGCGCGCCA-3'

BOTTOM: 3'-CGAATTAATTCCTAGGCCGCGCGGTGATC-5

(The PacI recognition sequence is TTAATTAA and that for AscI is GGCGCGCC). A 4kbp SalI fragment from plasmid pART27mod (containing the right border, lacZ marker (+multiple cloning site)nptII gene for kanamycin resistance under control of the *nos* promoter and polyadenylation signal and the left border) was cloned into the 13kbp vector pKT231 linearised with XhoI. Plasmid pKT231 carries the IncQ origin of replication for the host *Agrobacterium tumefaciens* and a resistance (marker) gene for streptomycin/spectinomycin. (Bagdasarian, M. & Timmis, K.N. (1982) Curr. Topics Microbiol. Immunol. **96**, 46-67). These two fragments were ligated using standard protocols (eg in Example No 3) and transformed into *E.coli* strain DH5α using standard protocols (eg in Example No 3). The resultant plasmid was named pJDML1.

2. Cloning HaSV genes into transfer plasmid

Construction of transfer vectors with HaSV genes:

Before the HaSV gene cassettes could be cloned into binary transfer vectors pART27 mod or pJDML1, they were re-cloned into the vector plasmid pBJ33 to provide flanking AscI and PacI sites. Plasmid pBJ33 (provided by Bart Janssen) is based on pBC SK(+) supplied by Stratagene), but with a multiple cloning site modified to contain the following sites:

SacI/PacI/AscI/SacII/Xbal/SpeI/BamHI/PstI/EcoRI/EcoRV/HindIII/ClaI/SaII/XhoI/ApaI/PacI/AscI/KpnI.

The cDNA fragment corresponding to complete HaSV RNA 1 behind the 35S promoter and terminating in the hairpin cassette ribozyme and the CaMV polyadenylation signal fragment (approx 6 kpb in total) was excised from plasmid pDHStuR1HC (Example 9) with EcoRI and cloned into EcoRI-cut vector pBJ33 to give plasmid pBJ33R1HC. Similarly, the cDNA fragment corresponding to complete HaSV RNA 2 behind the 35S promoter and terminating in the hairpin cassette ribozyme and the CaMV polyadenylation signal fragment (approx 3.3 kbp in total) was excised from plasmid pDHStuR2HC (Example 9) as two fragments, one (covering the 35S promoter and the first 500 bp of the RNA 2 sequence) of about 1kbp with EcoRI and R5rII and the second (covering the remainder of the RNA 2 sequence, the ribozyme and the polyadenylation signal) of about 2.3kbp with RerII and HindIII.

These two fragments were simultaneously ligated into EcoRI and HindIII-cut vector pBJ33 to give plasmid pBJ33R2HC.

A 1.9 kbp fragment comprising the 5' 1.7 kbp of the HaSV capsid gene, together with the polyadenylation fragment, were excised from expression plasmid pDHVCAPB (described in Example 5) as a Eco RI - KpnI fragment and cloned into pTZ19U (pharmacia) cut with EcoRI and KpnI, giving pTZ19UEVCAPB., This portion of the HaSV capsid gene expression cassette was then re-excised as a HindIII-EcoRI fragment and cloned into PBJ33 cut with these enzymes. This plasmid (pBJ33EVCAPB) was then linearized with EcoRI and the ca. 800 bp EcoRI fragment from pDHVCAPB carrying the 35S promoter and the 5' 250 bp of the capsid gene inserted, followed by screening for orientation. The resulting plasmid carrying the reassembled complete capsid gene expression cassette was named pBJ33VCAPB.

Assembling binary plasmids:

The RNA 1 expression cassette was excised from plasmid pBJ33R1HC with AscI and PacI and cloned into pART27 mod cut with AscI and PacI to give pMLR1. The RNA 2 expression cassette was also cloned as an AscI-PacI fragment into pJDML1 cut with AscI and PacI to give pJDMLR2.

The capsid protein gene cassette was excised from pBJ33 VCAPB with PacI and cloned into plasmid pMLR1 cut with PacI. Resulting plasmids were screened for orientation and the plasmid with the capsid gene and RNA1 in the same orientation was named pMLR1V. The complete fragment carrying the HaSV capsid gene and

RNA 1 expression cassettes in pMLR1V was excised with AscI and cloned into pJDMLR2 linearised with AscI to give pHaSV1 (29kpb). This plasmid carries the HaSV capsid gene expression cassette and the HaSV RNA 1 and RNA 2 expression cassettes in this order and all in the same orientation. The kanamycin resistance gene is located upstream of the capsid gene and in the opposite orientation.

Table of constructs generated:

	Vector	Insert(s)	Name	#Plants (independent transformants)	Comments
	pART27mod	RNA 1	pMLR1	15	control
	pJDML1	R1 + R2 +CAP	pHaSV1	30	complete virus
	pART27mod	R1 + CAP	pMLR1V	15	subvirus
	pJDML1	R1 + CAP	pJDMLR1V	30	subvirus
	pART27mod	RNA2	pMLR2	15	control
	pJDML1	RNA2	pJDMLR2	15	control
	pART27mod	CAP	pMLVF	15	control

(CAP = HaSV capsid gene)

3. Plant transformation and regeneration

Binary transfer vectors (above) were transformed into *Agrobacterium tumefaciens* strain LBA4404 by electroporation (Lin, J.J. (1994) FOCUS **16**,18-19; Lin, J.J. (1994)

Plant Science **101**, 11-15). Leaf discs from *Nicotiana tabacum* grown under sterile conditions were transformed using cocultivation with transformed *A. tumefaciens* (Horsch, R.B. *et al.* (1984) Science **23,** 496-498; Horsch, R.B. *et al.* (1988) Plant Molecular Biology Manual A5:1-9; as modified by Lisa Molvig (pers. comm.)) and grown on kanamycin. After transfer of regenerating shoots for further selection on kanamycin medium, kanamycin-resistant roots were selected and then tissue from these plants used to verify HaSV gene expression. The numbers of plants selected are shown in the table above for each of the constructs.

4. Western, Northern and Southern blotting on transgenic plants

For western blots: A small amount (0.1g) of fresh leaf material from each plant was extracted by grinding in 0.2 ml of plant extraction buffer (0.2M NaCl, 0.1M Tes, pH 7.65, 1mM PMSF, 2% b-mercaptoethanol, 1mM EDTA). After centrifugation to pellet plant debris the supernatant was collected and 10µl aliquots run on a SDS-gel for blotting and immuno-analysis with antibody against HaSV as described in Example 1. The results for the first plants assayed are given in Table 3.

For Northern blots: Total leaf RNA was extracted from 0.15 g of fresh leaf material. The leaf material was ground under liquid nitrogen to a powder and then extracted by further grinding in 0.45 ml NTES buffer (0.1 M NaCl, 10mM Tris-HCl pH 8.0, 1mM EDTA, 0.1% SDS) plus 0.45 ml Tris pH8.0-saturated phenol/chloroform. The slurry was vortexed, centrifuged for 3 min and the aqueous phase mixed with 1 volume of isopropanol to precipitate RNA and DNA. After resuspending the pellet in 0.1 ml

water, 1 volume of 4M LiC1 was added and the mix stood on ice overnight before centrifugation to pellet RNA. The RNA was then analysed by gel electrophoresis according to the methods in Examples 1 and 2. HaSV specific RNAs were detected by Northern blottings as described in Example 2 and by using riboprobes made to detect the 3'-terminal 1000 nucleotides of each of RNA 1 and 2, made using the Promega Riboprobe kit and used as specified by the supplier.

For Southern blots: to detect HaSV genes in plant genomic DNA.

To recover plant genomic DNA, the supernatant from LiCl precipitation (above) was mixed with 2 volumes of ethanol. The pellet was redissolved and the DNA cut with BamHI before analysis on agarose gels and transfer to nylon membrane as described by Sambrook *et al* (1989) and by the manufacturer (Zetaprobe/BioRad). HaSV-specific bands were detected described above.

5. Bioassays on leaf material

Two small leaves (2-3 cm in length) were selected from each transformed plant selected, and placed in petri dishes containing 1.5% agarose in water. Three to 8 neonate larvae were placed in each petri dish and observed for 3 days. At the end of this time, larvae were weighed and then total RNA extracted as described in Example 1. The extent of leaf damage was quantified by measuring the area of leaf consumed by each group of larvae over the three days of the assay (see Table 3).

Table 3

Preliminary bioassay of HaSV transgenic plants

Three to 8 larvae were placed on a small leaf (from a newly regenerated plant) in a petri dish with no provision of fresh food, after 3 days, larvae were sacrificed and northern blotted; also, protein extracted from leaves of the plants were western blotted using anti-HaSV antisera.

Plant	Transform-ation Plasmid	Western Blot for HaSV capsid protein in plant (+/-)	Northern blot for HaSV RNA in plant	Larval Weight (mg)	Leaf Damage (mm^2 consumed /larvae)
Negative Controls		-	-	2.1-2.7±0.8*	61
1.1 (subvirus)	pJDMLR1V	+		1.1±0.2	29
(RNA1=p71)					
3.2 (whole virus)	pHaSV1	+		1.0±0.4	38
3.4 (whole virus)	pHaSV1	+		1.2±0.4	32

* Diet was limiting (ran out of food) in some cases

Table 4

Further bioassay of HaSV transgenic plants

Four - 6 individual larvae were fed leaf disc ($50mm^2$) from control or transgenic plants at one disc each per day for 4 days, before transferred to artificial diet for a further 3 days. RNA was then extracted from the larvae and Northern blotting with HaSV-specific probes used to verify the presence of HaSV in the larvae.

Plant	Transformed with	Western blot for HaSV capsid protein in plants (+/-)	Mean larval weight (mg)
negative control		-	12.4
positive control (leaf + HaSV)		-	0.1
3.2	pHaSV1	+	0.9
3.10	pHaSV1	+	4.8
3.11	pHaSV1	+	8.2

Efficacy of HaSV as atransvirus in plants

Factors affecting the efficacy of HaSV are the viral dose required, the expression levels achieved in plants and the leaf damage observed. These need to be considered separately at this stage due to uncertainty about the efficiency of HaSV assembly in plants and because larvae will continue feeding for about one day after receiving a toxic dose of HaSV.

I. Dose of Virus

Infection with HaSV requires neonate larvae to eat up to 10 000 particles. Assuming that transgenic plants make only 1 particle per cell, this means the larvae must consume up to 10 000 leaf cells.

Since a small tobacco leaf contains about one million cells, larvae could acquire a toxic dose by consuming just 1% of the leaf.

This dose would correspond to as little as 0.000 000 5% of the soluble protein in these cells (330x 10^{-13} g of HaSV per leaf in $7x10^{-13}$g soluble plant protein per leaf).

II. Expression levels

Assuming standard levels of 1% expression and complete incorporation into virus particles, there should be about 10^8 particles per cell ($7x10^{-9}$g of protein per cell over $330x10^{-19}$g per HaSV particle).

However, at present only part of this protein is likely to form infectious virus. If 1% does, then there would be 10^6 particles per cell, well above the toxic dose.

Initial results from Western blots suggest current expression at least exceed 01.1% of soluble cell protein. Processing of the precursor protein appears to occur to a variable extent, suggesting that particle assembly has also occurred.

The dose of infectious virus delivered by transgenic plants must be quantified by appropriately standardised bioassays.

Optimisation of the infectious virus level will be achieved by improving virus assembly rather than just boosting expression of components - this represents a fundamental difference to the situation with toxins like Bt.

I. *Dose of Virus*

Infection with HaSV requires neonate larvae to eat up to 10 000 particles. Assuming that transgenic plants make only 1 particle per cell, this means the larvae must consume up to 10 000 leaf cells.

Since a small tobacco leaf contains about one million cells, larvae could acquire a toxic dose by consuming just 1% of the leaf.

This dose would correspond to as little as 0.000 000 5% of the soluble protein in these cells (330x 10^{-13} g of HaSV per leaf in $7x10^{-13}$g soluble plant protein per leaf).

II. *Expression levels*

Assuming standard levels of 1% expression and complete incorporation into virus particles, there should be about 10^8 particles per cell ($7x10^{-9}$g of protein per cell over $330x10^{-19}$g per HaSV particle).

However, at present only part of this protein is likely to form infectious virus. If 1% does, then there would be 10^6 particles per cell, well above the toxic dose.

Initial results from Western blots suggest current expression at least exceed 01.1% of soluble cell protein. Processing of the precursor protein appears to occur to a variable extent, suggesting that particle assembly has also occurred.

The dose of infectious virus delivered by transgenic plants must be quantified by appropriately standardised bioassays.

Optimisation of the infectious virus level will be achieved by improving virus assembly rather than just boosting expression of components - this represents a fundamental difference to the situation with toxins like Bt.

III. Leaf damage

While as little as 1% of the leaf (and more likely far less) may be sufficient to deliver a toxic dose of HaSV, larvae will keep feeding for a limited period after becoming infected. This makes it necessary to determine the extent of leaf damage empirically.

Our initial observations were that plants making detectable levels of HaSV capsids showed reduced susceptibility to larval feeding; this has not been quantified yet, and the assay was a severe one.

Consumption of leaf material by infected larvae may be estimated indirectly using our data on larval growth and frass production, which are approximately equal. Since neonate frass production is too low to quantify, the data were obtained from 4-day old larvae. These produce 30 mg of frass over 7 days, compared to 400 mg for uninfected controls. Neonate growth and frass production may be estimated at 10% of this figure.

Assuming that 1 mg growth or frass - 3 mg leaf material, an infected neonate will consume about 5% of a small tobacco leaf (20 mg of a total fresh weight of 350 mg) over seven days compared to over 60% for an uninfected control (240 mg of 350 mg).

Biosafety Considerations

It is believed that the approach of controlling pests by making an insect virus in transgenic plants is not dangerous to the environment. This is despite our very tentative observation that some HaSV replication is observed in protoplasts. There has been widespread debate recently concerning the safety of protecting crops against plant viruses by inserting transgenes expressing viral proteins into the plants. Falk, B.W. and Bruening, G., 1994 (Science **263**, 1395-1396) identified 3 possible mechanisms which might result in the appearance of novel viruses. These mechanisms are transencapsidation, phenotypic mixing and heterologous recombination.

Plasmids	Bioassays weights (mg) of larvae fed on protoplast extracts	HaSV RNAs 1 & 2 detected by Northern blotting of RNA extracted from larvae
1. pDHStuR1HC + pDHVCAPB	29 ± 15	+
2. pDHStuRI HDV + pHV CAPB	57 ± 25	(-)
3. Control: (diet only/diet + protoplasts	85 ± 15	-
4. pDHStuR1 HC + pDHStuR2 HC + pDHVCAPB	33 ± 28	+
5. pDHStuR1 HDV + pDHStuR2 HDV + pDHVCAPB	64 ± 22	-

ii) RNA extraction from larvae showed

(a) that larvae fed protoplasts transfected with pDHStuR1HC + pDHStuR2HC + DHVCAPB contained both RNA1 and 2 of HaSV in intact form.

(b) that larvae fed protoplasts transfected with pDHStuR1HC + DHV CAPB (subvirus) contained a very small amount of intact HaSV RNA1 and a considerably greater amount of degraded RNA1.

(c) that larvae fed protoplasts transfected with pDHStuR1HDV + pDHStuR2HDV + pDHVCAPB contained no HaSVRNA with one exception.

(d) that larvae fed protoplasts transfected with pDHStuR1 HDV + pDHVCAPB contained no HaSV RNA.

Conclusions:

The HC (HaSV expression) constructs with the hairpin cassette ribozyme give infectious particles with both RNAs; the HDV expression constructs do not under these conditions.

That the subvirus approach results in RNA1 replicating in larvae but this RNA is degraded because it cannot be encapsidated due to missing replicatable RNA2.

That subvirus approach gives stunting as effectively as does the complete virus approach under these conditions.

EXAMPLE 12

CAPSOVECTOR

The aim of this section is to describe the invention of capsovectors and present supporting data. In addition ideas for their further improvement will be presented.

The capsovector is a virus-like particle (VLP) from a small RNA insect virus with a pest insect host whose properties will facilitate the entry (or vector) into the cells of the pest insect either, or both, RNA or a protein that will induce toxicity in the insect. Capsovectors can be produced in transgenic crop plants targeted by the host insect pests or produced for spray applications by transgenic plants or recombinant microorganisms.

There are two types of capsovectors, ones that vector RNA moieties (RNA capsovectors) and ones that vector proteinaceous moieties (protein capsovectors). Because of their distinct properties, RNA and protein capsovectors will be dealt with separately. However, the description of the protein capsovector will rely heavily on the preceding description of the RNA capsovector.

In this invention, the *Helicoverpa armigera* stunt virus (HaSV) of the Tetraviridae will be used as the model insect small RNA virus. However, this does not exclude other types of insect small RNA viruses being used in a similar manner.

Characteristics of HaSV pertinent to capsovectors.

HaSV is a member of the Tetraviridae family of viruses and infects only midgut cells of young larvae of heliothine insects (Hanzlik et al., 1993) after ingestion with food. Numerous attempts to grow the virus in non-gut tissue, other insects and cultured cells have failed. This believed to be due to the ability of the HaSV capsid protein to bind and enter only midgut cells of the host insect (Hanzlik et al., 1995).

HaSV has been characterised in great detail at the molecular level. Its physical characteristics have been determined (Hanzlik et al., 1993) and its complete genome sequenced (Gordon, et al., 1995; Hanzlik, et al., 1995).

particles is aimed at replication of new virions replication with any toxicity is a by-product of this process; that contained within capsovectors is aimed at rapid toxicity to the cell and not at replicating new capsovectors.

Important to the toxic activity of a RNA capsovector are the properties of viral genes that lead to the activities of encapsidating, protecting, entry, uncoating and translating of the viral RNA in the host cell. These activities are necessary for successful virus infection yet do not involve replication. Because of the simplicity of HaSV, all of these properties are contained in one gene and its product, the coat protein, p71. Generally, the coat protein gene of other small RNA viruses have the same capacity and therefore are also suitable for capsovectors. The p71 gene of HaSV is employed in illustrating the capsovector invention and its properties are explained and demonstrated under their respective headings below.

The capsovector adds toxicity to the virus functionalities by using sequences exogenous to HaSV or viruses on the RNA contained within the capsovectors. These toxic sequences are aimed at inducing rapid and direct toxicity to the cell the capsovector has entered. The sequences are either translated into protein or fold the RNA into appropriate secondary structures which then causes a toxic lesion in the cell. These sequences are explained below as well.

It must be pointed out here that viruses avoid inducing rapid and direct toxicity to cells they have entered as this is deleterious to viral replication. Because the cell must be viable for the production of new viruses, many viruses do not induce cell death until late in their infections if at all. Indeed, infection of hosts by many insect small RNA viruses different from HaSV does not result in any discernible response in the host insect upon its being infected. These innocuous viruses can still be exploited, however, because the viruses are able to perform the previously mentioned functions of encapsidation and protection of the labile RNA genome, ability of the particles to enter host cells, uncoating and subsequent translation of the RNA genome. Appropriate placement of toxin sequences with those of the coat proteins of these viruses as described in this invention can result in toxicity and subsequent control of the insect pest host.

Encapsidation. Encapsidation is defined here as the process of forming a virus particle or a virus-like particle that incorporates RNA into its interior. For this process

to occur, interactions among the capsid proteins (encoded by the RNA) and between the proteins and specific regions of the RNA must occur that result in the ordered aggregation of 240 copies of the coat protein and the RNA strand(s). The specific regions on the RNA are either or both a defined primary sequence or a specific secondary structure arrived at by a number of primary sequences.

All three types of interactions, protein/protein, RNA/protein and RNA folding into secondary structures, reside in the capsid gene ORF of HaSV. This is shown by the following data:

1. When the ORF the HaSV capsid protein is expressed in insect cells with a recombinant baculovirus, VLPs can be observed with transmission electron microscopy (TEM) in sections of fixed, positively-stained, infected cells. They are highly similar in morphology and dimensions to native virions observed in gut tissue of diseased insects. The VLP morphology is that of a smooth surfaced sphere with a diameter of 35-40 nm identical to virus particles observed in diseased tissue. Also noted is that a fraction of the particles have dark, electron dense cores. The fraction of VLPs having electron dense cores is smaller than that observed for virus particles observed in diseased tissue. The electron dense cores indicate that the particles contain electron dense, non proteinaceous RNA.

   Methods and Materials: A recombinant baculovirus able to express p71 was made by the following procedure. An amplicon containing the p71 ORF was obtained from a PCR reaction made with HP64NEUK (GGCCGGATCCAGACATGCTGGAGTGGCGTCAC) and HVP6C2 (GGGATCCCTAATTGGCACGAGCGGCGC) off a DNA template consisting of the pT7T2p71 plasmid used for the bacterial expression of the capsid gene. This fragment contains the p71 ORF flanked with BamHI sites and the initiating AUG placed behind a more favourable context for expression in eukaryotes. This fragment was restricted with BamHI and cloned into the baculovirus expression vector, pVL941, which was transfected into Sf9 cells with linearized wildtype baculovirus DNA. A recombinant baculovirus, Bacp71, was obtained by standard means (King and Possee, 1992).

   For TEM, Sf9 cells infected for three days with Bacp71, were harvested, fixed with gluteraldhyde, embedded in LRWhite resin and sectioned for

examination with TEM using standard procedures. The sections were examined with a JEOL 100CX transmission electron microscope.

2. When the VLPs are purified and characterised, they also show highly similar characteristics to native HaSV virions. Buoyant densities of the particles are similar 1.296 g/ml for virions and 1.29 g/ml for VLPs. Particle morphologies as seen by negative stained TEM are highly similar with the micrographs for both virions and VLPs showing spheres of 35-40 nm spheres possessing electron dense interiors. The electron dense interiors also indicate the presence of RNA.

Methods and materials. After a three day infection with Bacp71 in a 220 cm^2 flask, Sf9 cells were harvested, pelleted, washed, and lysed with a freeze-thaw cycle in 10 ml of buffer. This was centrifuged at 10,000 x g for 10 min and the supernatant was recovered and recentrifuged on top of a 30% sucrose cushion. The pellet was then redissolved in 0.5 ml of buffer (50mM Tris pH7.5, 5 mM CaCl) overnight and placed on top of a solution in a SW41 tube (Beckman) consisting of 5 mls each of 60% and 30% of CsCl in buffer. This was centrifuged overnight at 40,000 rpm and the sole band located in the middle of the tube was removed, placed into 10 ml of buffer and pelleted by centrifugation at 35,000 rpm in a SW41 rotor. The pellet was dissolved in 50 m l of buffer.

VLPs in the solution were examined on a JEOL 100CX microscope with standard procedures after negative staining with uranyl acetate..

3. The VLPs contained RNA. The above observations indicated that the VLPs in the were electron dense and that RNA was within the VLPs. These observations were confirmed when the VLPs were extracted for RNA and the RNA analysed by agarose gel electrophoresis. The RNA was 2900 bases in length, the expected size of the mRNA transcribed from the baculoviral genome. When the RNA was probed with a radioactively labelled probe specific for p71 sequences, strong hybridisation occurred, showing that the RNA was the p71 mRNA.

Methods and materials. RNA was removed from the purified VLPs with extraction with phenol, phenol/CH3Cl and CH3Cl and ethanol precipitation. This was then run on an 1% formaldehyde agarose gel. A northern blot of the gel was done with standard procedures. The ^{32}P-

labelled probe was prepared from clone HR326 which contains the p71 sequence.

4. The p71 mRNA extracted from the VLPs are shown to have heterologous, non-HaSV sequences. This is shown by the recovered RNA strand having a length of 2900 bases which is equal to the predicted size of the mRNA transcribed from the baculoviral genome and expressing the p71 ORF. Only 1900 bases of the mRNA are sequences from the p71 ORF, the rest being sequences from the baculovirus expressing the gene. When a radioactively labelled probe specific for the polyhedrin gene located between the p71 gene the polyadenylation signal for the polyhedrin gene was hybridized to the RNA extracted from the VLPs, a strong hybridation signal was seen on the autorad. This shows that the signals required for encapsidating RNA are present in the p71 ORF and that non-HaSV sequences can be placed inside the VLP.

Methods and materials. RNA extracted from VLPs were northern blotted by standard procedures and probed with a labelled 950 bp Hind III fragment from the baculoviral transfer vector pVL941 having sequences 3' to the inserted p71 gene and 5' to the polyadenylation site of the polyhedrin gene.

5. The RNA having the capsid protein ORF is specifically encapsidated. No other RNA except for the p71 mRNA is present in the VLPs. This is shown by the failure of another highly transcribed region of the baculoviral genome (therefore also present in great abundance inside the baculoviral infected cell) failing to hybridise to the VLP RNA. When a probe specific for the p10 mRNA, a late gene product from the baculovirus, is hybridised to the RNA extracted from the purified VLPs, no signal occurs on the northern blot.

Methods and materials. RNA extracted from VLPs were northern blotted by standard procedures and probed with a ^{32}P-labelled 245 bp Xba I/Eco RI fragment from the baculoviral transfer vector pAcUW31 having sequences 3' to the start of transcription of the p10 promoter for AcMNPV baculovirus.

Thus RNA sequences contained within the ORF of HaSV capsid protein (p71) can produce VLPs that encapsidate only RNAs having the p71 sequence. If there are exogenous sequences not from HaSV also on the p71 mRNA these then can be

encapsidated. This shows that sequences for toxins can be placed within the VLP if it possesses a p71 sequence.

Protection. Normally labile RNA contained within the capsovector must be protected from degradation during the period between its encapsidation inside the cell where it was produced and its entry into the cell where it will effect toxicity. This is particularly important because part of this period is spent in the insect gut that is a highly degradative milieu for both RNA and protein. This function is performed by the 240 copies of the capsid protein which form a protective shell around the virion RNA.

The protective properties of the HaSV coat protein for both the virion and the VLP was shown by western blots of the capsid protein from HaSV particles, HaSV VLPs, and lipophorin before and after timed exposure to the gut contents of heliothis larvae: The data shows that:

1. When a non-viral globular protein like lipophorin is exposed to the contents of the heliothis midgut, rapid degradation of the protein occurs within 10 minutes.
2. When either an HaSV virion or VLP is exposed in the same manner, minimal degradation of the protein occurs despite extended exposure times (>2 hr).

Thus, when translated, the RNA sequences contained within the p71 ORF lead to protection of the protein and RNA of the VLP.

Methods and materials. The midguts of fifth instar Heliothis armigera were excised, their contents removed and centrifuged at 14,500 x g for 15 min. Into 10 ml of the contents were placed 1 ml of a solution having 1 mg protein of either lipophorin, HaSV virions or HaSV VLPs. At timed intervals 1 ml of this solution was removed and immediately boiled in SDS-PAGE sample buffer. SDS-PAGE and immunoblots with the respective antisera were then carried out with standard procedures.

Cell Entry. Studies of animal host/virus systems have shown that entry of virions into cells is mediated by cellular receptors located on their exteriors and viral acceptor proteins or VAPs (Lentz, 1990). The HaSV VAP is by exclusion of any other possibility, p71, .it being the sole protein component of the HaSV capsid (Hanzlik et al., 1993, 1995). In particular, it is believed that a specific region of p71, between residue 274 and 439 of the protein sequence, is responsible for the binding of HaSV to the presently unknown host cell receptor.

entry into a new host cell. The maturation cleavage is mediated by the encapsidated RNA in the interior of the capsid (Wery et al., 1994). The maturation cleavage displays itself in HaSV by the appearance of a 64 kDa protein and the disappearance the 71kDa precursor similar to the nodaviruses (Gallagher and Rueckert, 1988)).

That this process occurs for HaSV VLPs is shown by observing an immunoblot of extracts of cells infected with Bacp71 and expressing p71 and proteins extracted from purified HaSV virions and purified HaSV VLPs. The blot shows that for the cell extract, p71 is expressed in the majority with minor expression of lower molecular weight products that are presumed degradation products. For proteins extracted from HaSV virions, the 64 kDa cleavage produce is present in the great majority with only a minor presence of the 71 kDa precursor. For proteins from VLPs, the 64 kDa and 71 kDa proteins are present approximately equal amounts showing that the maturation cleavage does occur although not as efficiently as with viral RNA present inside the particles.

Thus HaSV VLPs are in a condition to be uncoated when they enter a host cell. This process is mediated by RNA sequences in the coat protein ORF.

Methods and materials. An extract of cells infected with Bacp71 for three days was made by pelleting cells from a 25 cm^2 flask and lysing them in phosphate-buffered saline with freeze-thaw. SDS-PAGE and immunoblotting with anti-sera against HaSV was performed on the cell extract and proteins from VLPs and virions according to standard procedures.

Translation. As a general rule, ribosomes responsible for translating proteins initiate translation by binding to the cap structure of an mRNA then scanning to the first MET placed in an appropriate context. This presents an initial difficulty in translating a toxin sequence on an RNA from a protein product (the VLP) translated from the region of the mRNA where the encapsidation signal resides (see above).

This difficulty can be dealt with in either of two ways: trans-encapsidation or through the use of internal ribosome entry sites (IRESs).

1. Trans-encapsidation. Trans-encapsidation is the process whereby an RNA strand is produced with the toxin sequence placed in good translatable context before the p71 encapsidation sequence. Translation of an RNA sequence is not required for its encapsidation by proteins produced from another mRNA. In this way a VLP

produced from an RNA having a translatable p71 encapsidates an RNA with a nontranslatable p71 sequence but possessing the encapsidation signal that ensures the encapsidation of the strand with a translatable toxin sequence.

2. IRESs. IRESs are one of the exceptions to the general rule noted above for translation They are sequences on an RNA strand that allow ribosomes to bind an RNA internally instead of at the 5' cap to initiate protein translation. They are located in the 5' regions of picornavirus vRNAs and specialised genes from certain organisms (Jackson et al., 1994)). They have been employed to allow the translation of two proteins from the same mRNA (Lipsick and Smarda, 1993) and thus can be employed to express a toxin from a sequence located. after a translatable p71. An advantage to this approach is that many IRESs are host specific. If the IRES used in the RNA capsovector is able to function only in the target organism, the toxin is produced only in the target organism and not in the organism producing the RNA capsovector. A picornavirus with a targeted pest insect host will possess a suitable IRES for a capsovector. For example, an IRES from a picornavirus with a Heliothis host will be used in a capsovector constructed with p71 from HaSV and a cytotoxin such as the ricin A fragement. This particular capsovector will affect only heliothine insects and no others as well as not producing a ricin A fragment in the plant producing the capsovector.

Translation into Toxicity.

Ultimately, all of the above abilities of encapsidation, protection, entry, and uncoating must be induced or accomplished by sequences in the RNA strand that produce the capsovector. Also important are sequences on the RNA, derived from other viral and non-viral sources as well as from HaSV, that are responsible for the toxic activity and if required, translation into protein that confer the toxicity. These types of sequences will be dealt with in separate sections.

RNA sequences leading to toxicity of the organism or cell can either be translated into protein or the sequences cause secondary structures to made on the RNA strand that lead to toxicity.

1. Toxicity from protein sequences. Both types of toxins, nerve toxins, specific for insects and work at the level of the organism (Binnington and Baule, 1993) and cytotoxins (Stripe and Barbeiri, 1986), toxic only to the cell the capsovector has entered, can be used with RNA capsovectors. However, the former type of toxin will

have to have a secretion signal appropriate to midgut cells. Examples of these proteins are described in Maeda et al., 1991.

2. Toxicity from RNA secondary structure sequences. RNA secondary structures will have the ability to cause toxicity to the cell to which they have been vectored by RNA capsovectors. These structures are caused by primary sequences; however, any number of primary sequences on the RNA strand can lead to the same toxic secondary structure. There are three types of these sequence structures that will be appropriate for RNA capsovectors: antisense sequences, ribozymes and mimicking structures. The first two types are reviewed by Eguchi et al., 1991 and elsewhere herein, respectively and aimed at preventing the expression of key cellular enzymes. The latter is novel and will be detailed.

- It is the activity of the HaSV replicase and not virosis or accumulation of viruses that causes the midgut cell to cease functioning. This is shown by data generated from the following experiment. When protoplasts are transfected with genes that make a replicatable RNA1 and only the capsid protein and not a replicatable RNA2 (R1-HC and VCAPB according to procedures listed above), stunting occurs. When the stunted larvae are extracted for RNA which is then northern blotted with probe for HaSV nucleic acid, only RNA1 of HaSV is seen to be present. Stunting does not occur when the protoplasts are transfected with genes that do not make a replicatable RNA1 (lacking an effective ribozyme to cleave after the last viral base in the gene) and only the capsid protein and not a replicatable RNA2 (R1-HDV and VCAPB according to procedures listed elsewhere in patent). When the stunted larvae are extracted for RNA which is then northern blotted with probe for HaSV nucleic acid, no HaSV RNA is seen to be present.

- These data are consistent with RNA1 being encapsidated and able to enter midgut cells of the larvae. RNA1 is able to self-replicate but not produce virions as there is no replicatable RNA2 which has the p71 ORF. The self-replication leads to antibiosis due to apoptosis of the midgut cells having the replicating RNA1. The particles made in the same manner but having RNA1 not able to self-replicate (due to the 3' sequences left by the defective ribozyme) were unable to stunt the larvae.
- As shown in other systems, the activity of the replicase may be mimicked by placing a by-product of replicase activity into the cell. This action "tricks" the cell into initiating anti-viral measures such as shut-down of protein synthesis or cell death by apoptosis (the activity believed to be responsible for the antibiosis caused by HaSV).

- One such by-product of the replicase's activity is double stranded RNA, an intermediate of RNA replication of tetraviruses (du Plessis et al., 1991). Other systems have shown that transfection of double stranded RNA into cells causes anti-viral measures to be initiated in them.
- Double-stranded RNA can be delivered to midgut cells by RNA capsovectors by a synthetic gene construct which produces a large stem-loop structure when transcribed. This structure is made by making the 3' half the reverse complement of the 5' half which then self-hybridises into the stem-loop. The RNA can be of viral, from either HaSV strand or of non-viral origin.

Protein Capsovectors.

Protein capsovectors are VLPs composed of a modified capsid protein or of a mixture of the modified capsid protein and the unmodified capsid protein. The modified coat protein is the coat protein, p71, fused to a fragment of a cytotoxin of either a plant or bacterial origin. When expressed, the coat proteins and coat protein-toxin fusions will self-assemble into the protein capsovector. Similar to the RNA capsovectors, protein capsovectors will not be self-replicating entities like viruses. Upon being eaten by an insect pest, the structure of the capsovector will vector the toxin moiety to inside the midgut cell by preventing proteolysis of the toxin moiety in the midgut and entering the midgut cells in a manner similar to what occurs for a virus particle. Upon entry, the capsovector will expose the cytotoxic moiety which will then kill the cell. Large numbers of midgut cells killed by capsovectors will cause antibiosis to the feeding insects. It is believed that a single gene will be able to express the capsovector.

In concept, protein capsovectors are similar to the immunotoxins successfully used in human cancer therapy where cytotoxic moieties are vectored to specific cells by fusion to a specific binding moiety such as antibodies or cytokines. By themselves, the cytotoxin fragments do not display toxicity even when injected intravenously. Only when they are attached or fused to a binding element does cytotoxicity occur to those cells to which the element binds. The binding element of the protein capsovector is the VAP part (see above) of the HaSV VLP which is able to bind and enter midgut cells of heliothis larvae.

However, the protein capsovector is distinct from immunotoxins in that its structure also protects the toxin moiety from degradation in addition to binding to the midgut

cell. The toxin fragment will be contained within the capsid shell until the capsovector enters the midgut cell. Also, it is the capacity of capsovectors to protect the toxin moiety from degradation in the midgut lumen that makes it distinct from other insect control factors that are fused to elements that only "interact" with midgut cells.

In the following sections, each of the two elements of a capsovector, the cytotoxic fragment and a viral coat protein gene, will be described followed by descriptions of how the capsovector are constructed with data supporting their feasibility.

The toxin. Several cytotoxin fragments suitable for protein capsovectors are available in readily accessible form. , They have extensive literature describing their activity in various fusions for use in immunotoxins (Thorpe et al., 1982). For expression in plants, plant-derived fragments of proteinaceous toxins, such as ricin A fragment, which are not toxic to plant cells but toxic to animal cells will be the most suitable. For expression in microorganisms, toxins of bacterial origin may be the most suitable, it being that they are not toxic to the microorganism producing the protein capsovectors.

As described in the section on RNA caspsovectors, p71 has the ability to self-assemble into VLPs when expressed in various non-host expression systems. Also shown was the ability of the VLPs and virions to resist degradation. and bind to, then enter, midgut cells. The critical question concerning the feasibility of the protein capsovector using p71, the HaSV coat protein, is the ability of VLPs to form with the modified coat proteins fused to toxins.

Construction of model protein capsovectors with capsid protein, p71 and a reporter peptide. The cytotoxin fragment can be fused to p71 in a number of ways. In order to test the possibilities, a reporter peptide fragment was used in place of the toxins. This allows a more rapid characterisation of the products as immunodetection of the exogenous fragment is facilitated by a commercially available monoclonal antibody specific for the fragment (IBI FLAG Biosystem). Two constructs were made using standard techniques. The FlagT construct placed the reporter fragment, sized 2501 Da, at the C-terminus of p71 (Fig. 8). The FlagM construct placed a reporter fragment, sized 1243 Da, in the middle of the p71 sequence near the site where p71 is cleaved into p64 and p7 (Fig. 8). Both constructs should have resulted in the reporter fragment being placed into the interior of the VLP where p7 is located.

Formation of VLPs made with modified p71. Recombinant baculoviruses were made with the modified p71 genes by standard techniques (King and Possee, 1992) and used to infect Sf9 cells. When these cells were processed and examined as before with TEM, particles highly similar to VLPs made with unmodified p71 and HaSV virions were observed. The particles were purified on CsCl gradients and were examined with SDS-PAGE, immunoblots, negatively stained TEM. In addition, the particles were tested for their ability to protect the FLAG epitope on the fused peptide fragment. These experiments showed:

1. The purified particles were highly similar to HaSV virions and VLPs made with unmodified p71 and showed the characteristic 35-40 nm diameter spheres with a fraction having the electron dense cores. The FlagT particle possessed a buoyant density of 1.31 g/ml compared to 1.29 g/ml for unmodified VLPs and virions.
2. Proteins extracted from the particles and observed with SDS-PAGE had the molecular weights predicted from the constructs (1243 Da and 2501 Da for FlagM and FlagT respectively. In addition, post-assembly cleavage into p64 was observed for the FlagM particle. No processing was seen in the FlagT particle.
3. Proteins extracted from the particles reacted with both the anti-p71 antisera and with the FLAG monoclonal antibody on immunoblots.
4. The FLAG epitope was protected during exposure to heliothis midgut contents. This is shown by the FLAG epitope remaining at the original molecular weight and therefore undegraded.

Hybrid VLPs. Although VLPs can be made with only modified p71 fused to the reporter peptide, and protection of the exogenous reporter peptide occurs, a protein capsovector made with both native p71 and modified p71 fused to a cytotoxin may function better. At present it is not clear what properties of the "native" VLP, if any, are altered with the addition of the exogenous, fused peptides to p71. If any deleterious properties arise such as poor stability of the particle, a resolution to the problem will be to produce a hybrid particle. This will minimise any disruption of desirable properties of the native VLP by the modified p71.

Hybrid VLP expression in plants. Three ways can be envisioned to express, either in a transgenic plant, the hybrid capsovector which requires two distinct, but closely

related proteins. The most obvious is two insert two genes into the plant, one for each protein. However, the use of elements from plant viruses can make it possible to express a capsovector from a single gene. These elements are suppressible stop contexts and frame-shift sequences that are detailed by Sleat and Wilson (1992). The use of these elements make it feasible to precisely regulate the ratio of coat protein to coat protein-toxin fusion.

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